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Interactions of the local anesthetic tetracaine with glyceroglycolipid bilayers: a ^{2}H-NMR study *

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We have examined the effects of the local anesthetic tetracaine on the orientational and dynamic properties of glycolipid model membranes. We elected to study the interactions of tetracaine with the pure glycolipid 1,2-di-*O*-tetradecyl-3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol (β-DTGL) and a mixture of β-DTGL (20 mol%) in dimyristoylphosphatidylcholine (DMPC) by deuterium NMR (^{2}H-NMR) spectroscopy. ^{2}H-NMR spectra of β-DTGL have been measured as a function of temperature in the presence of both the charged (pH 5.5) and uncharged forms (pH 9.5) of tetracaine. The results indicate that the anesthetic induces the formation of non-lamellar phases. Specifically, the incorporation of uncharged tetracaine results in the formation of a hexagonal phase which is stable from 52 to 60 °C. At lower pH, the spectrum at 52 °C is very reminescent of that of the β-glucolipid alone in a bilayer environment, while as the temperature is elevated to 60 °C, a transition from a spectrum indicative of axial symmetry to one due to nearly isotropic motion or symmetry occurs, which may result from the formation of a cubic phase. Although it leads to an alteration in the phase behavior, the presence of tetracaine does not induce large changes in the headgroup orientation of β-DTGL. In contrast to the pure glycolipid situation, the interaction of tetracaine with β-DTGL (20 mol%) in DMPC does not trigger the formation of non-lamellar phases, but leads to a slight reduction in molecular ordering. The presence of the charged form of the local anesthetic near the aqueous interface of the bilayer appears to induce a small change in the conformation about the C2-C3 bond of the glycerol backbone of β-DTGL in the mixed lipid system. Thus, the major influence of the local anesthetic on glycolipids is a change in the stability of the lamellar phase, facilitating conversion to phases with hexagonal or isotropic environments for the lipid molecules.

Introduction

Local anesthetics are known to exert their action by blocking the sodium channels of nerve membranes. However, whether this blocking is a result of a direct anesthetic–protein interaction [1] or a perturbation by the anesthetic of the lipid matrix surrounding the channels [2] is still unclear. The interactions of the local anesthetic tetracaine with phospholipid model systems have been the subject of several studies. Deuterium nuclear magnetic resonance (^{2}H-NMR) is a powerful technique to obtain information on both the degree of order and the molecular dynamics of liquid-crystalline media and has been extensively used on model and natural membranes [3–5]. Results obtained by ^{2}H-NMR [6–9] suggest that tetracaine interacts differently with different phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), depending mostly on the charge and shape of the lipid studied. Moreover, both ^{2}H-NMR and high-pressure Fourier-transform infrared (FT-IR) studies [8,9,10,11] have shown that the charged form of the local anesthetic tetracaine at pH 5.5 is located close to the aqueous interface of pure phospholipid bilayers while the uncharged form at pH 9.5 penetrates deeper into the bilayer. In the case of the charged form of tetracaine, several NMR studies have also shown that the anesthetic interacts with phospholipid headgroups [8,12].

A natural extension of these studies with model systems is the study of the interactions of local anesthetics with unmyelinated and myelinated nerves [7,11]. In the case of myelinated nerves, the myelin sheath surrounding the nerve assumes considerable importance in maintaining proper nerve function. Thus in addition to probing the direct effect of agents on nerves, it is of

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interest to ask if it is possible that the same agents can lead to indirect effects through perturbation of the organization and physico-chemical properties of myelin.

Glycolipids are common components of the myelin sheath, in particular a glycosphingolipid, galactocerebroside is present. The influence of various agents on the physico-chemical properties of phospholipids, also components of myelin, has been studied extensively. However, the glycolipid components have received much less study. Thus, it is of interest to probe the effects of these agents on other components of myelin. The orientational and motional properties of an analog of the neutral myelin glycolipid, the glyceroglycolipid, 1,2-di-*O*-tetradecyl-3-*O*-*β*-glucosylglycerol (*β*-DTGL), have been studied by ^{2}H-NMR spectroscopy [13,14] and are well characterized. Since this glycolipid has been shown to be a reasonable model for the corresponding glycocerebroside [15], we have examined the effect of the local anesthetic tetracaine on the physical properties of the *β*-DTGL headgroup. We have studied the interaction of the local anesthetic with pure *β*-DTGL and with the glycolipid (20 mol%) in dimyristoylphosphatidylcholine (DMPC), a situation which more closely resembles that in the myelin sheath.

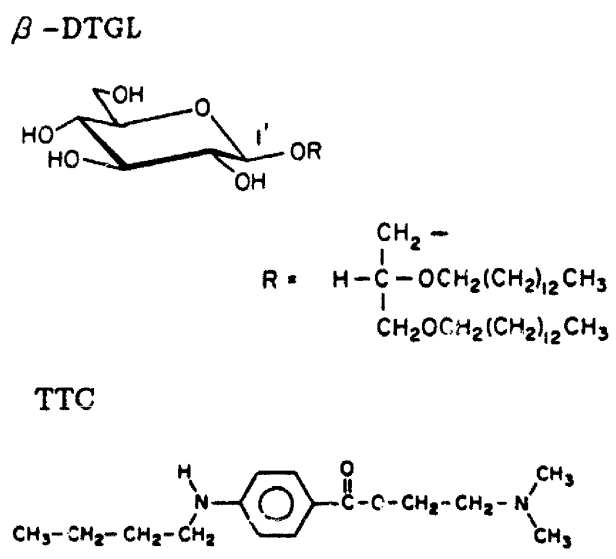

Fig. 1. Fig. 1. Structure of 1,2-di-*O*-tetradecyl-3-*O*-*β*-glucosylglycerol (*β*-DTGL) and tetracaine (TTC).

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) and tetracaine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO. 1,2-Di-*O*-tetradecyl-3-*O*-(*β*-D-[1-^{2}H$_1$]glucopyranosyl)-*rac*-glycerol and 1,2-di-*O*-tetradecyl-3-*O*-(*β*-D-glucopyranosyl)-*sn*-[3,3-^{2}H$_2$]glycerol were prepared as described previously [13,14]. Deuterium-depleted water used for all samples was obtained from Aldrich Chemical Co., Milwaukee, WI; all other materials were analytical grade. NMR samples were prepared by hydrating 100 mg (0.14M) of total lipids (pure *β*-DTGL and *β*-DTGL (20 mol%) in DMPC) with 1 ml of buffer containing about 10 mg of anesthetic (0.033 M). The pure *β*-DTGL samples consisted of 20% of labelled lipid in unlabelled glycolipid. The sample was then subjected to at least 10 freeze-thaw cycles to ensure complete equilibration of the anesthetic [16] Samples prepared in this manner gave very reproductible results. The buffer (BPC) consisted of sodium citrate (0.02 N) sodium phosphate (0.02 N), sodium borate (0.017 N), and sodium chloride (0.1 N) [17] in deuterium-depleted water in order to minimize the HDO signal.

^{2}H-NMR data were acquired at 30.7 MHz on a 'home-built' spectrometer operated by a Nicolet 1280 computer. Spectra were acquired by means of a modified quadrupolar echo sequence [18]; quadrature detection was used to record the echo signals. The pulse spacing was typically 60 μs, the $\pi/2$ pulse length was 3.8 μs (10-mm solenoid coil) and the recycle time was 100 ms ($> 5T_{1Z}$). Samples were enclosed in a glass jacket where the temperature was regulated to within $\pm$ 0.5C°. The spectra, which have lineshapes indicative of axial symmetry, were 'dePaked' according to Bloom and co-workers [19] to obtain the 90° oriented-sample spectra and the quadrupolar splittings.

Results and Discussion

Interaction of tetracaine with pure β-DTGL

The ^{2}H-NMR spectra of *β*-DTGL, labelled at C-1′ of the glucosyl moiety and C3 of the glycerol backbone, at 52°C in the presence and absence of tetracaine at pH 9.5, where the anesthetic is primarily uncharged, are shown in Fig. 2. Previous studies have shown that *β*-DTGL exhibits a transition from a gel- to liquid-crystalline phase at 52°C and from a lamellar to a hexagonal phase at 58°C [13]. In the absence of tetracaine at 52°C, the spectrum is characteristic of axially symmetric motion attributable to the lamellar phase. This spectrum persists up to 58°C. At 60°C (results not shown), the ^{2}H-NMR spectra retain the shape characteristic of axially symmetric motion but the quadrupolar splittings are reduced by more than a factor of two, suggesting a hexagonal aggregate structure for the lipid. If all motional and orientational parameters remain the same as in the lamellar phase, the quadrupolar splitting is expected to be reduced by a factor of two for the hexagonal-phase lipid relative to that of the lamellar phase [3–5]. The fact that the quadrupolar splittings at 60°C are reduced by more than one-half the values at 52°C suggests that the orientation of the sugar ring is changed relative to that in the lamellar phase.

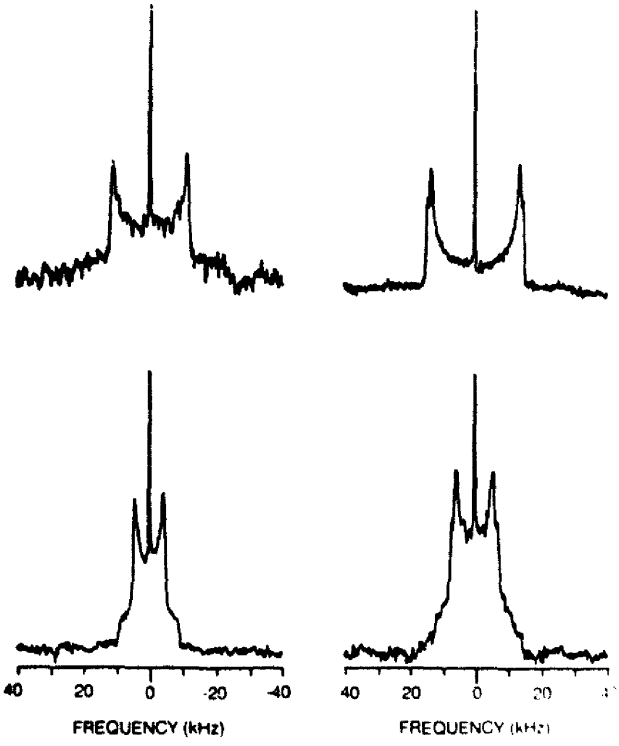

Fig. 2. ^{2}H-NMR spectra (30.7 MHz) of β-DTGL labelled at C-1′ of the carbohydrate headgroup (left) and at C-3 of glycerol (right) in the absence (top spectra) and presence of tetracaine (bottom spectra) at pH 9.5, 52°C.

In the presence of uncharged tetracaine, and for temperatures from 52 to 60°C, the quadrupolar splitting of β-DTGL labelled at C-1′ of the carbohydrate headgroup is also reduced by more than a factor of two, suggesting that, as with pure β-DTGL at higher temperature (58°C), the lipid is in the hexagonal phase. However, with these data alone, one cannot discount a conformational change in the headgroup. To establish which of the two possibilities is occurring, β-DTGL labelled at the C-3 position of the glycerol backbone was examined (Fig. 2, right). A reduction in the quadrupolar splitting of the system in the presence of tetracaine is seen. The parallel behaviour of two very different positions in the lipid is consistent with a hexagonal aggregate structure of the lipid, which is stable from 52 to 58°C. Such destabilization of the lamellar phase of a glycolipid by exogenous molecules has previously been demonstrated [20,21].

It has been shown that the transition between lamellar and non-lamellar phases in lipid/water model systems is affected by temperature, hydration, cations, pH and additions of exogenous molecules such as steroids, alcohols, local anesthetics and detergents, as well as the structure of the lipid molecules [21]. The formation of different lipid aggregate structures is dependent on the cross-sectional area of lipid at the hydrocarbon/water interface, the hydrophobic volume and the hydrocarbon chain length of the participating molecules. Lamellar phases are built-up of cylindrical-like molecules. However, a decrease in the interfacial area of the lipid, and an increase in the hydrophobic volume, gives the lipid molecules the effective shape of a truncated cone. This shape favors the formation of non-lamellar aggregates of the reverse type (water in oil), such as the hexagonal H_{II} phase.

Previous ^{2}H-NMR [8,9] and high-pressure FT-IR [10,11] studies have shown that the uncharged form of the local anesthetic tetracaine at pH 9.5 partitions deeply into pure phospholipid multilamellar dispersions, whereas the charged form at pH 5.5 remains close to the headgroup region. If the uncharged form of tetracaine partitions relatively deeply into the hydrocarbon region of the glycolipid bilayer, as it does for pure phospholipid bilayers, the requirement for the formation of a hexagonal phase is satisfied. This is supported by the fact that nonpolar organic solvents that partition more or less deeply into the hydrophobic interior of the bilayer [22,23] are able to transform a lamellar phase into non-lamellar phases of the reversed type. For example, the presence of *n*-dodecane in the systems egg phosphatidylethanolamine/water [24] and dioleoyl-PE (DOPE)/dioleoylphosphatidylcholine (DOPC)/water [25], which normally form a L_α phase, indcues the formation of a H_{II} phase. These results are therefore in agreement with the induction of a hexagonal phase of the reverse type in β-DTGL by the addition of the uncharged form of the local anesthetic tetracaine.

At lower pH (5.5), where the anesthetic is primarily charged, a dramatically different picture is obtained, as shown in Fig. 3. At 52°C, the ^{2}H-NMR spectrum is very similar to that obtained for the β-glucolipid alone, labelled in either the headgroup or the glycerol positions, and is attributable to a lipid in a lamellar phase. This contrasts with the corresponding system at pH 9.5 in which a non-lamellar structure occurs at the same temperature. As the temperature is elevated to 60°C, the spectra indicate a transition from an axially symmetric to a nearly isotropic state for the lipids. The total integrated intensity of the spectra remains constant, within experimental error, indicating that the observed changes are not attributable to the effects of very short transverse relaxation times, T_{2e}. The spectrum due to isotropic behaviour may result from several possibilities, including formation of vesicles, micelles, or macroscopic structures having high symmetry. Visual inspection of the sample at 60°C clearly indicates that micelles or small vesicles are not the dominant structures present. It has been shown that in addition to forming hexagonal phases, glycolipids can form macroscopic structures of high symmetry, such as cubic and rhombic, and that exogenous molecules can facilitate the formation of such structures [20]. It appears that for the β-glucolipid, the presence of tetracaine close to the aqueous interface

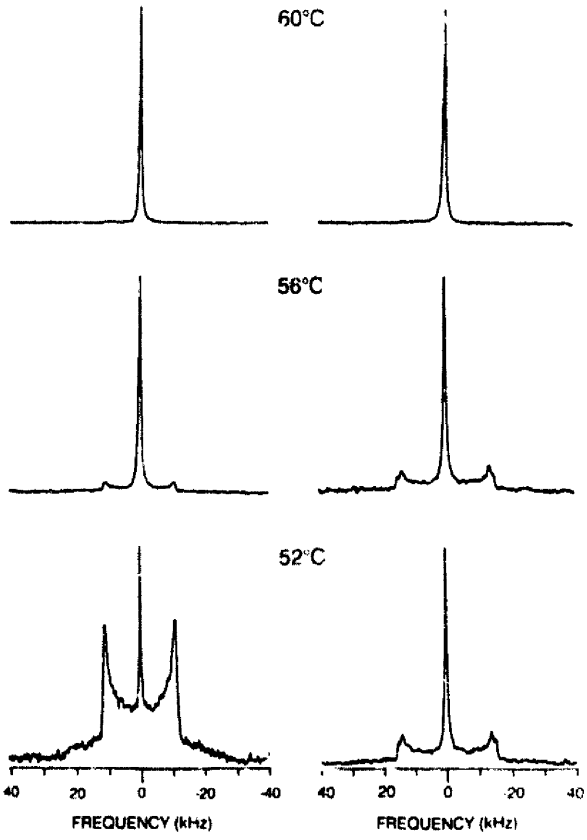

Fig. 3. Temperature dependence of ^{2}H-NMR spectra (30.7 MHz) of [1'-$^2H_1$]β-DTGL (left) and [3,3-$^2H_2$]β-DTGL (right) in the presence of tetracaine at pH 5.5.

of the bilayer induces, over the temperature range of 52 to 58°C, the formation of a cubic or rhombic phase.

The transition from lamellar to a continous cubic structure has been rationalized by Lindblom and co-workers [26] in the following way. In a sample of fixed composition, an increase in temperature will increase the chain mobility toward the chain end, resulting in a tendency to form a structure where the cross-sectional area per molecule at the methyl end groups is larger than at the polar headgroups. The continuous cubic structure proposed by these authors would result in a larger cross-sectional area per molecule at the methyl end group compared to the cross-section of the polar headgroup. ^{2}H-NMR [8,9] and high-pressure FT-IR [10,11] studies have shown that the local anesthetic tetracaine, primarily charged at pH 5.5, is located close to the aqueous interface of pure phospholipid bilayers. If this is so for tetracaine partitioned into β-DTGL bilayers, then the presence of tetracaine close to the aqueous interface of the bilayer may induce, over the temperature range of 52 to 58°C, the formation of a continuous cubic phase. In this system, the cross-sectional area at the methyl end group would be greater than that for the pure β-DTGL molecule, since tetracaine would act as a spacer between the lipid headgroups. A transition from a lamellar to a cubic phase has also been observed by Cullis and co-workers [27] for a diphosphatidylglycerol system in the presence of the local anesthetic dibucaine. An additional feature of the transition from a lamellar to an isotropic phase for β-DTGL in the presence of charged tetracaine is that, on cooling the sample from 60 to 52°C, the formation of a lamellar phase does not readily occur. Such a hysteresis effect has been reported for other systems undergoing aggregate structural changes [28].

While the spectral changes presented in Figs. 2 and 3 for β-DTGL in the presence of both the charged and uncharged forms of tetracaine may be attributed to lamellar-to-non-lamellar transitions, information on headgroup orientation may also be sought. For a C-^{2}H bond executing axially symmetric motion the quadrupolar splitting is given by [29]:

$$\Delta\nu_Q = \frac{3e^2qQ}{4h}\left(\frac{3\cos^2\alpha_i - 1}{2}\right)\left[\frac{3\cos^2\theta - 1}{2}\right] \quad (1)$$

where e^2qQ/h is the quadrupolar coupling constant, α_i is the angle between the C-^{2}H bond vector and the axis about which the molecular segment undergoes rotation and the term in square brackets describes the motion of the rotation axis. The latter term is referred to as the segmental order parameter, S_{mol}, and describes the anisotropic motion of the entire rigid structure.

The quadrupolar splittings for deuterium in both the headgroup and glycerol moieties at pH 9.5 in the presence of tetracaine at 52°C are very similar to those of β-DTGL alone at 58°C (hexagonal phase). This indicates that both molecular ordering and headgroup orientation are essentially the same for both of these systems. For β-DTGL in the presence of charged tetracaine at 52°C, the quadrupolar splittings for both labelled positions are nearly identical to those obtained for the pure glycolipid at 52°C (lamellar phase), again reflecting similar orientational and motional properties of the glycolipid headgroup. The headgroup orientation of β-DTGL is therefore not significantly altered by the presence of the local anesthetic tetracaine. In contrast, the lamellar structure of the glycolipid system becomes less stable in the presence of tetracaine.

The behaviour of the glycolipid system in the presence of anesthetics is very different from that of phospholipids. ^{2}H-, ^{31}P- and ^{14}N-NMR studies [8,12] have shown that the charged form of the local anesthetics tetracaine and dibucaine interacts with phospholipid headgroup. Specifically, the absolute magnitude of the ^{31}P chemical shift anisotropy increases from a value of 48 ppm to 58 ppm in the presence of tetracaine while the ^{14}N quadrupolar splitting decreases. The ^{2}H quadrupolar splittings of the C_α and C_β positions of the phosphatidylcholine headgroup are also changed in the

presence of charged anesthetic, the binding of tetracaine or dibucaine decreasing the C_α splitting and increasing the C_β position splitting. Similar effects have been observed with metal cation binding to the phosphatidylcholine headgroup [30,31], although the magnitude of the effects caused by anesthetics is much larger. However, at higher pH (9.0), the magnitude of the effect of tetracaine on phosphatidylcholine headgroup is much smaller than that at low pH [8]. This demonstrates the deeper penetration of the uncharged form of tetracaine in PC bilayers.

The interactions of phospholipid polar groups with charged or dipolar molecules have recently been rationalized in terms of the electrical properties of the membrane surfaces [32]. These studies demonstrated that phospholipid headgroups are sensitive to electric surface charges and that the conformational change of the phosphocholine dipole with positive charges is inverse to that occurring with negative surface charges. For a given charged molecule, the quadrupolar splitting is linearly related to the amount of bound/adsorbed molecules and only small rotations are sufficient to induce large variations in the quadrupolar splittings [31,32]. In the case of the neutral glycolipid investigated in this study, the electrical properties of the membrane surface will be different than those of zwitterionic phospholipid membranes; as a result, conformational changes in the β-DTGL headgroup in the presence of charged tetracaine are negligible.

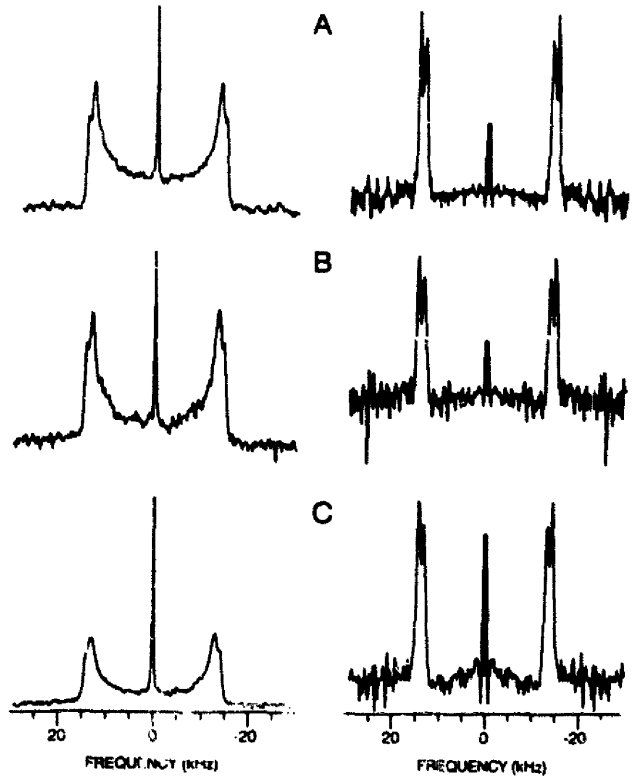

Fig. 4. ^{2}H-NMR spectra (30.7 MHz) (left) and dePaked counterparts (right) of [3,3-$^2H_2$]β-DTGL in: (A) DMPC/β-DTGL, (B) DMPC/β-DTGL + TTC (pH 9.5) and (C) DMPC/β-DTGL + TTC (pH 5.5), all at 50 °C. TTC, tetracaine.

Interaction of tetracaine with β-DTGL (20 mol%) in DMPC

In contrast to the pure glycolipid system, the interaction of tetracaine with β-DTGL (20 mol%) in dimyristoylphosphatidylcholine (DMPC) at both pH 5.5 and 9.5 does not lead to the formation of non-lamellar phases. The ^{2}H-NMR spectra of β-DTGL, labelled in both headgroup and glycerol positions, in the presence of tetracaine closely resemble those obtained in its absence. Specifically, the quadrupolar splitting for the C-1′ labelled positions of the glucose ring is about 20 kHz, both in the absence and presence of tetracaine (spectra not shown).

The ^{2}H-NMR spectra of [3,3-$^2H_2$]β-DTGL (20 mol%) in DMPC in the absence and presence of tetracaine (pH 5.5 and pH 9.5) are shown in Fig. 4, together with the corresponding dePaked spectra. These spectra exhibit two quadrupolar splittings, indicating that the two C-^{2}H bonds at this positions make different angles with respect to the axis of motional averaging [14]. In the presence of both the charged (pH 5.5) and uncharged (pH 9.5) form of tetracaine, there is a small decrease of the quadrupolar splittings associated with the two inequivalent deuterons, this effect being slightly more pronounced at pH 5.5. For example, the largest quadrupolar splitting at 37 °C is reduced from 30.5 kHz to 29.7 kHz and 29.3 kHz in the presence of uncharged (pH 9.5) and charged (pH 5.5) tetracaine, respectively. These effects are small but constant over the whole temperature range studied (37 to 50 °C). This decrease in the quadrupolar splittings can be related to a change in one or both angular term of Eqn. 1. One possibility implies a change in the orientation of the glycerol backbone relative to its motional axis, which would then result in a change of the angle α (Eqn. 1) for the two deuterated positions. On the other hand, a small reduction of the segmental order parameter S_{mol} can also account for the decrease in the quadrupolar splittings. In order to discriminate between the two possibilities, the ratios of the observed quadrupolar splittings for the two deuterons of the glycerol backbone were calculated. Since S_{mol} is constant for this segment of the molecule, this ratio is sensitive to changes in the angle α for each deuteron [29].

If the average orientation of the C-3 position of glycerol remains unchanged upon addition of tetracaine, the ratio should stay constant for the different systems. This is in fact observed for the three systems studied, which indicates that the average orientation of the C-3 position of glycerol is unchanged by the pres-

ence of tetracaine in DMPC/β-DTGL (4 : 1) bilayers. The reduction of the quadrupolar splittings observed in the presence of tetracaine can thus be related to a decrease of the segmental order parameter S_{mol} by 5%. These reductions are small but constant over the temperature range studied and indicate that the presence of tetracaine causes a small reduction in segmental order.

Inspection of the dePaked spectra in Fig. 4 reveals that the two quadrupolar patterns for the C-3 position in β-DTGL have different component widths. Previous studies from our laboratory [14] have demonstrated that this additional line broadening is due to ^1H-^2H dipolar coupling between the proton at C-2 and the two deuterons at C-3. If the C2–C3 segment of glycerol is considered, this dipolar coupling is dependent on the conformation about the C2-C3 bond. Oriented sample spectra of [3,3-$^2H_2$]β-DTGL obtained with ^1H decoupling showed that the two quadrupolar splittings have the same linewidth [14]. From these results, the conformation about the C2-C3 bond of glycerol was estimated.

In the presence of uncharged tetracaine at pH 9.5 (Fig. 4B), the width of the two quadrupolar splittings for the C-3 position in β-DTGL are very close to that obtained for pure β-DTGL, the inner doublet being slightly broader than the outer one. This suggests that the conformation about the C2-C3 bond is not significantly changed by the addition of the uncharged anesthetic. However, in the presence of charged tetracaine at pH 5.5 (Fig. 4C), the outer doublet appears to be slightly broader than the inner one. With ^1H decoupling, the widths of the two doublets for this system become essentially equal, as was observed for the pure glycolipid system [14]. These results suggest different couplings $^1H2$-$^2H3$ (S) and $^1H2$-$^2H3$ (R) (S and R referring to the pro-S and pro-R deuterons of C3) for β-DTGL in the presence of charged tetracaine. The conformation about the C2-C3 bond of the glycerol for β-DTGL (20 mol%) in DMPC may therefore be altered by the presence of charged tetracaine. Since the charged form of tetracaine in phosphatidylcholine bilayers interacts relatively strongly with the phospholipid headgroup [8], it is of interest to note that a change in DMPC headgroup orientation induced by charged tetracaine may cause the change in the conformation about the C2-C3 bond of the glycerol backbone of β-DTGL, a change that is not observed at higher pH. The results are further supported by the fact that unlike the orientation of the C2-C3 bond of the glycerol backbone which is relatively invariant from lipid to lipid, the conformation about this bond appears to vary significantly [14,33]. Additional labelling of the glycerol moiety would however be required to enable a more precise definition of the glycerol conformation for β-DTGL (20 mol%) in DMPC in the absence and presence of tetracaine.

Conclusions

The present study demonstrates that unlike the interaction of tetracaine with phospholipids, there is no large change in the headgroup orientation of β-DTGL. The stability of the lamellar structure of the pure glycolipid system is very sensitive to the presence of anesthetic while the mixed glycolipid-phospholipid systems exhibit only a lamellar structure.

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