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Rapid report

Sphingolipid-derived signalling modulators: interaction with phosphatidylserine

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Abstract

We previously described the synthesis of two deuterium-labelled sphingoid bases, which made it possible to perform NMR spectroscopy on this family of signalling modulators in membranes (Rigby, A.C, Barber, K.R and Grant, C.W.M. (1995) Biochim. Biophys. Acta 1240, 75–82). In the present work we sought to test the concept that such mediators may display altered physical behaviour through association with anionic lipids – as a possible mechanism of involvement in signal transduction. Lyso-dihydrogalactosylceramide with deuterium nuclei at C_4 and C_5 of the sphingosine backbone and at C'_3 and C'_4 of the galactose ring ([$^2H_4$]lyso-GalCer), and *N,N*-dimethylsphingosine with deuterated amino-methyl groups ([$^2H_6$]dimethylsphingosine), were assembled as minor components into unsonicated fluid bilayer membranes of 1-palmitoyl-2-oleoylphosphatidylcholine/cholesterol. The effect of (anionic) phosphatidylserine was considered in this zwitterionic host matrix. The results present a picture of rapidly reversible interaction. The (−) charged phosphatidylserine exerted readily-measurable control over the orientation of the carbohydrate residue of [$^2H_4$]lyso-GalCer. In contrast, surrounding (−) charges exerted little spectral influence at the level of C_4 and C_5 of the lyso-GalCer, membrane-inserted, backbone; or at the level of the amino group of dimethylsphingosine. It would appear that packing alterations induced by the phosphatidylserine/sphingoid base association can translate into sizeable spatial constraints in the neighbouring aqueous domain.

Keywords: Dimethylsphingosine; Glycosphingolipid, lyso-; NMR, ^{2}H-; Ceramide; Bilayer

In recent years an important involvement in signalling has emerged for sphingolipid breakdown products in higher animal cells [1–5]. It was noted early on that the major group of these compounds share the structural feature of being (+) charged by virtue of the presence of the amino group characterising sphingoid bases [6,7]. Hence the possibility that their role in signalling pathways may involve interaction with other (oppositely charged) membrane lipids has attracted considerable attention [1,3]. In particular, workers have pointed out the possibility of interaction between (+) charged sphingoid bases and (−) charged phosphatidylserine [1,6–10] (see also [11] for related considerations involving diacyl glycerol pathways). Alteration in lipid arrangement or packing, associated with the opposite charges of sphingoid bases vs. phosphatidylserine, has been proposed as a mechanism for control of protein kinase C [9,12,13].

In order to understand relevant charge-related interactions, the phase behaviour of sphingosine in bilayers of neutral and acidic phospholipids has been characterised [9,10]; as has its ability to influence electrostatic interaction of large molecules with acidic model membranes [8]. Local structural implications of chemical groups in sphingolipid breakdown products have been examined via their effect on monolayer collapse pressures [9,14]. In the present work we made use of the fact that ^{2}H nuclei provide non-perturbing probes of lipid conformation and motional characteristics [15–17] to measure the physical consequences of sphingoid base interaction with phosphatidylserine.

Unsonicated bilayers of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) containing 33 mol% cholesterol were employed as the host membrane. The zwitterionic POPC is often considered adequately representative of common membrane phospholipids in terms of fatty acid

Abbreviations: GalCer, dihydrogalactosylceramide; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; lyso-GalCer, lyso-dihydrogalactosylceramide; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine.

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composition. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylserine (POPS) was incorporated at levels consistent with maximal natural cytoplasmic leaflet concentrations. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylethanolamine (POPE) was used as a counterpart not having a net (−) charge. Lyso-galactosylceramide, which has been demonstrated to inhibit protein kinase C in cellular systems [18], was selected as a carbohydrate-bearing breakdown product of a relatively simple glycosphingolipid. In this regard, species with chain saturation at C_4,C_5 (dihydro species) are common naturally-occurring metabolites, which have been found to possess similar physiological effects on PKC to analogous sphingoid bases that are unsaturated at this location [1]. However it should be noted that the sphingenine analogue predominates naturally, and certainly some biological functions [1] and physical characteristics [14] of sphingoid bases can be sensitive to subtle chemical alterations. Dimethylsphingosine was chosen as a deglycosylated species that has been thoroughly characterised with regard to structural detail as a modulator of protein kinases in an epidermoid cell line in which it is the predominant sphingosine [19,20], and as an effector of platelet [21] and neutrophil metabolism [22]. These species were studied at between 5 and 10 mol% in various host matrices.

Phospholipids were obtained from Avanti Polar Lipids, Birmingham, AL. [$^2H_4$]Lyso-dihydrogalactosylceramide ([$^2H_4$]lyso-GalCer) was produced from deuterated dihydrogalactosylceramide (derived from natural beef brain galactosylceramide) [23,24]. It was confirmed by ^1H high resolution NMR in 98% DMSO/2% D_2O to have complete deuteration of the double bond, and variable but highly specific deuteration at C'_3 and C'_4 of the sugar ring. *trans*-D-Erythro-2-amino-4-octadecene-1,3-diol-4-sphingenine (D-sphingosine) isolated from bovine brain cerebrosides was obtained and converted to the deuterated dimethyl species via a modification of the method of Igarashi et al. [20], as described previously [24].

Lipid bilayer membranes for these experiments were prepared following a general protocol described elsewhere [24] and suspended in 5–30 mM Hepes (pH 7.0–7.2) containing 20 mM NaCl and 5 mM EDTA. Total lipid used per sample was typically 60–120 mg. ^2H-NMR spectra were acquired at 76.7 MHz on a Varian Unity spectrometer using a single-tuned Doty 5 mm solenoid probe, with temperature regulation to ±0.1 C° [24].

Chemical structures of the deuterated sphingolipid metabolites studied in the present work are shown in Fig. 1. [$^2H_4$]Lyso-GalCer has four sites of deuteration, which proved to be spectrally inequivalent. [$^2H_6$]Dimethylsphingosine has six deuterons (two deuterated methyl groups (-CD_3) attached to the nitrogen atom), which proved to be spectrally equivalent and produced only a single resultant Pake doublet. There has been some discussion regarding pK values for sphingolipid degradation products in membranes, however in general they are expected to be importantly protonated at physiological pH [6,10,19,24,25]. López-Garcia et al. found an apparent pK of 9.1 for sphingosine, which is a primary amine as is lyso-GalCer, in phosphatidylserine bilayers [10]. Tertiary amines such as dimethylsphingosine have higher pK values (discussed in [19]). Thus it seems likely that in our systems the (+) charged sphingoid base form is the predominant one near pH 7 [24] (Fig. 1).

Effects of interaction with charged lipids in membranes may be considered in some detail for [$^2H_4$]lyso-GalCer by examination of the typical wideline NMR spectra displayed in Fig. 2. These spectra reflect molecules undergoing rapid axially symmetric motion about axes perpendicular to the plane of the (fluid) bilayer membrane. Like all spectra encountered for this sphingolipid metabolite in the present work, they share the same three identifiable Pake doublet features (arrows in Fig. 2). Quantitative differences in spectral splitting were seen, dependent upon host matrix and upon location of the nuclear probe (Table 1).

Raney-nickel-catalysed addition of two deuterons across the double bond at C_4C_5 in the sphingosine backbone accounted for two deuteration sites in [$^2H_4$]lyso-GalCer. Exposure to Raney nickel is also known to lead to significant hydrogen/deuterium exchange at C'_3 and C'_4 of the GalCer sugar ring [23]. Under the conditions used, the

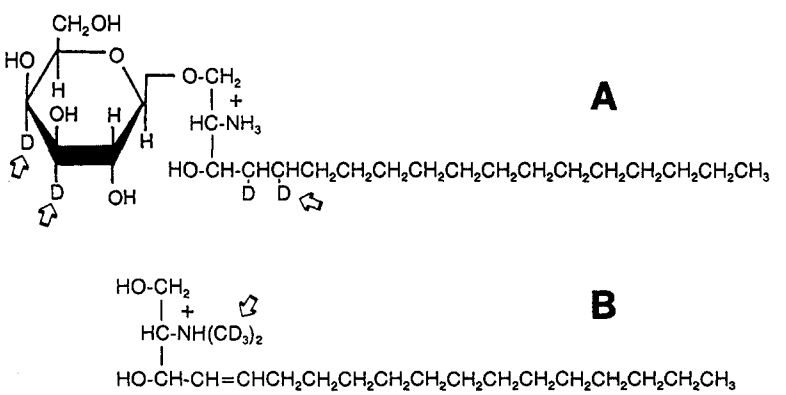

Fig. 1. Chemical structures of the selectively deuterated glycosphingolipid metabolites, [$^2H_4$]lyso-galactosylceramide (lyso-GalCer) (A), and [$^2H_6$]dimethylsphingosine (B). Arrows indicate the sites of deuteration, 'D', referred to in the present work.

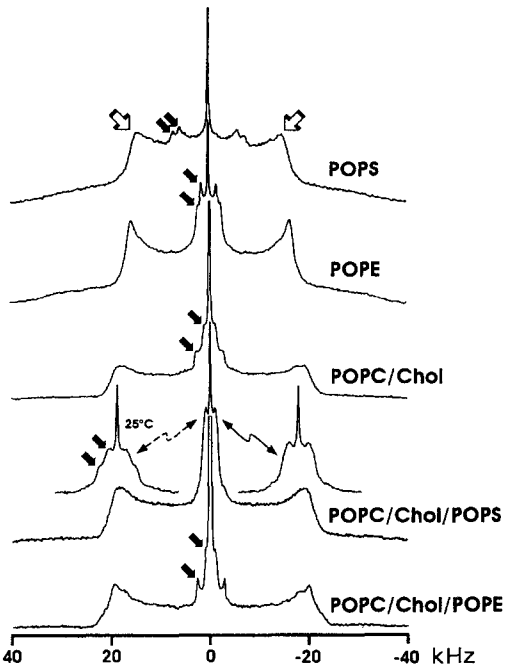

Fig. 2. Typical ^{2}H-NMR spectra for lyso-GalCer deuterated by complete hydrogenation within the alkyl chain at C_4C_5 and partial exchange at C'_3 and C'_4 of the sugar ring ([$^2H_4$]lyso-GalCer). In each case the deuterated lipid was dispersed as a minor component (5–10 mol%) in unsonicated host matrices of phospholipids: final sample pH was 7.2–7.3, temperature 37°C. Spectra are identified by host matrix: from top to bottom, pure POPS; pure POPE; POPC/cholesterol (2:1, mol ratio); POPC/cholesterol/POPS (2:1.5:1, mol ratio); POPC/cholesterol/POPE (2:1.5:1, mol ratio). The intense outer Pake doublet (large open arrows in top spectrum) was assigned to both deuterons introduced at C_4C_5 and is clearly identifiable in each spectrum. The (two), less intense, inner Pake doublets (small paired solid arrows shown only on the left side of each spectrum) were assigned to the (incompletely deuterated) sugar ring positions, C'_3 and C'_4. Note that these latter two peaks were difficult to resolve from one another for POPC/cholesterol/POPS at 37°C. However, peak location was readily confirmed by lowering the temperature: this is demonstrated in the insets above the spectrum (right hand inset is expanded frequency axis at 37°C, and left hand inset shows the same 12 kHz region at 25°C).

exchange reaction of ^{2}H into the sugar is less complete than hydrogenation of the double bond (see below). Four non-equivalent deuterons should give rise to four Pake doublets – while in fact only three doublets are immediately apparent in each spectrum (e.g. arrows in uppermost spectrum). Thus the intense outer Pake doublet (open arrows) was assigned to both deuterons introduced at C_4C_5. Such a result is expected if the average orientation of the sphingosine chain long axis is similar to that typical of intact glycerolipids and glycosphingolipids in membranes: it would then reflect the well-known 'plateau' of host phospholipid fatty acid order near the membrane surface [15–17,26–29]. That is, with the exception of deuterons at C_2 of fatty acids attached to the amino function of glycosphingolipids [30] or to the *sn*-2 position of glycerolipids [31], methylene deuterons near the membrane surface all give very similar, relatively large, splittings in a particular lipid under a given set of conditions.

The relationship governing Pake doublet 90°-edge quadrupole splitting ($\Delta\nu_Q$) for a given deuteron is as follows: [15–17].

$$\Delta\nu_Q = 3/8(e^2Qq/h)S_{mol}(3\cos^2\Theta_i - 1) \quad (1)$$

where e^2Qq/h is the nuclear quadrupole coupling constant (165–170 kHz for an aliphatic C-D bond), S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the lipid molecule relative to the bilayer normal, and Θ_i is the average orientation of each C-D bond relative to the molecular long axis.

The inner, less intense, Pake doublets seen in spectra of [$^2H_4$]lyso-GalCer (Fig. 2) arise from the smaller numbers of deuterons introduced by exchange at C'_3 and C'_4 of the galactose ring. Deuteration sites and extent of deuteration were verified by high resolution ^{1}H-NMR in DMSO, following the approach of Cioffi and Prestegard [23]. More prolonged deuteration times led directly to increased inten-

Table 1
Quadrupole splittings for spectra of [$^2H_4$]lyso-GalCer and [$^2H_6$]dimethylsphingosine in a variety of host matrices at 37°C

Host matrix	Spectral splittings ($\Delta\nu_Q$)		
	[$^2H_4$]lyso-GalCer		[$^2H_6$]dimethyl-sphingosine (±0.2 kHz)
	backbone deuterons C_4,C_5 (±0.5 kHz)	sugar deuterons C'_3,C'_4 (±0.2 kHz)	
POPC/Chol	39.0	5.9, 2.5 (2.4 [a])	2.4
POPC/POPS/Chol	39.6	3.0, 2.0 (1.5 [a])	2.5
POPC/POPE/Chol	40.5	5.7, 2.1 (2.7 [a])	2.5
POPS	31.1	15.0, 12.3 (1.2 [a])	2.0
POPE	33.3	4.9, 3.3 (1.5 [a])	1.9

POPC/cholesterol bilayers were used as a reference membrane. Spectral assignments for [$2H_4$]lyso-GalCer are indicated in Fig. 2. Where samples contained both POPC and POPS (or POPC and POPE) the mol ratio of POPC to POPS (or POPE) was 2:1. In all samples the mol ratio of total phospholipid to cholesterol was 2:1.

[a] Numbers in brackets give the outer-to-inner ratio of carbohydrate deuteron splittings. Variation in this number from sample to sample indicates a change in carbohydrate orientation.

sity in the Pake doublets assigned to the sugar ring deuterons, without affecting the rest of the wideline spectrum or the splittings measured (data not shown). These ^{2}H wideline NMR intensity increases were accompanied by the expected corresponding disappearance of sugar resonances from the lyso-GalCer ^{1}H high resolution NMR spectra. The sharp centre peak in each spectrum of Fig. 2, about which the above-mentioned powder patterns are symmetric, reflects the presence of some vesicles with high curvature for which the quadrupole splittings are motionally averaged to zero, and of residual HOD.

Spectra in Fig. 2 provide insight into molecular events that accompany lyso-GalCer association with PS in cell membranes. Mixtures of POPC with cholesterol are thought to be homogeneous and fluid in the temperature range studied here [32]. The centre spectrum in combination with the one immediately below it demonstrate the effect of incorporating maximal quantities of the cationic lipid, POPS, that might be found physiologically at the plasma membrane inner surface (20 mol%). The lowest spectrum in Fig. 2 illustrates a comparable sample in which zwitterionic POPE was substituted for POPS in an otherwise identical sample. Measured ^{2}H-NMR quadrupolar splittings, $\Delta\nu_Q$, corresponding to these and other selected samples are listed in Table 1.

The informative parameter, $\Delta\nu_Q$, for deuterons at C_4 and C_5 in the hydrophobic interior can be seen to be, at most, modestly affected by the presence of these quantities of POPS or POPE. Thus the spectral splitting of 39.0 ± 0.5 kHz for lyso-GalCer in POPC/cholesterol was changed to 39.6 ± 0.5 kHz or 40.5 ± 0.5 kHz for the same mixture containing 20% POPS or POPE, respectively (Table 1). Alterations in the splittings associated with C'_3 and C'_4 in the carbohydrate portion were also small in the case of added POPE, but quite striking in the case of added (charged) POPS. Moreover, the ratio of the carbohydrate spectral splittings changed from 2.4 in POPC/cholesterol (2.7 with added POPE) to 1.5 with added POPS. Changes in this ratio demonstrate that the charged lipid effect was associated with reorientation of the sugar headgroup on its sphingosine backbone (Eq. (1) dictates that alterations to S_{mol} would leave the ratio of the ring deuteron splittings unchanged). The effect seen is reminiscent of the 'molecular voltmeter' phenomenon first noted by Seelig and coworkers [33]: that surface charge alters quadrupole splittings associated with the deuterated headgroup of phosphatidylcholine in membranes.

Spectra of deuterated lyso-GalCer dispersed as a minor component in pure POPS and pure POPE membranes are included in Fig. 2. Although highly unnatural in lipid composition, these membranes serve to illustrate the magnitude of changes that might accompany specific association of lyso-GalCer with PS in the above more natural samples. For lyso-GalCer dispersed in pure POPS vs pure POPE the spectral splittings associated with deuterons at C_4C_5 in the hydrophobic domain were 31 and 33 kHz, respectively (Table 1, uppermost two spectra in Fig. 2). These values represent reduction in the splittings by 21 and 18%, respectively from the values observed in the presence of cholesterol. The primary effect in each case seems therefore to be removal of the well known ordering effect of cholesterol [34]. In contrast, markedly different effects of (−) vs. zwitterionic lipid were seen in the sugar headgroup: splittings of 15.0 and 12.3 kHz for POPS vs 4.9 and 3.3 kHz for POPE. Clearly, in the pure POPS matrix the magnitude of the spectral splitting change is much greater than that seen in the host matrix where the predominant lipids are POPC and cholesterol. These observations suggest that charge is important, but that major phase separation of POPS/lyso-GalCer domains is not occurring in the POPC/cholesterol/POPS lipid matrix.

We have not assigned which of the two splittings attributed to C'_3 and C'_4 of the galactose residue is associated with which of these deuterons (solid arrows in Fig. 2). The spectra obtained from the deuterated sugar residue in [$^2H_4$]lyso-GalCer are closely related to spectra first reported by Skarjune and Oldfield for glucosylceramide deuterated in the sugar ring [35], and to those of similarly deuterated glyceroglycolipids [36–38]. However, splittings seen for the sugar ring deuterons of lyso-GalCer are several times smaller than the largest ones reported for sugars on intact glycolipids. These small quadrupole splittings would be consistent with reduced carbohydrate motional order resulting from absence of the glycolipid fatty acid in lyso-GalCer. However proof of this rationale would require additional information to separate order (S_{mol}) from orientation (Θ_i) in Eq. (1). Membrane surface implications of removal of the glycosphingolipid fatty acid are very complex [14]. The parent compound of [$^2H_4$]lyso-GalCer (identically-deuterated [$^2H_4$]GalCer) demonstrated a dramatic spectral difference in that the deuterons associated with the carbohydrate residue produced nonaxially symmetric spectral features with intensity maxima at 6 and 21 kHz, consistent with greater motional restriction. We have observed nonaxially symmetric spectra for galactosylceramide deuterated in the sugar hydroxymethyl group in the POPC/cholesterol host matrix [39]; and as here, the motions responsible for the spectral asymmetry appeared to be associated with the headgroup portion of the glycolipid.

Powder spectra of [$^2H_6$]dimethylsphingosine in our membrane systems were characterised by a single Pake doublet having a small quadrupole splitting (1–2 kHz). We previously demonstrated that this single Pake doublet reflects both deuteromethyl groups, which are expected to be spectrally equivalent as a result of rapid stereoisomer interconversion [24]. In the present work, for two dozen samples having different combinations of surrounding host matrix, including pure POPC, POPS, POPE and combinations with cholesterol, the splitting remained within the range, 2 ± 0.5 kHz. Relevant examples are included in Table 1. Note for instance that at 37°C the spectra for

dimethylsphingosine in POPC/cholesterol, with or without POPS or POPE was a doublet having a quadrupole splitting near 2.5 kHz. The largest change in this value was seen when cholesterol was removed from the matrices – causing the spectral splitting to drop by about 1 kHz, and presumably reflecting reduced motional order as noted above for lyso-GalCer. A very similar spectrum has been reported for deuterated methyl groups in phosphatidylcholine – a single Pake doublet with splitting of 0.9 kHz [40,41]. Our results are consistent with highly disordered motion and/or with the deuterated methyl group average orientation being by chance near the magic angle of 54.7°. Either way, there is little evidence of sensitivity to host matrix (−) charge.

The presence of non-perturbing deuterium probes on the sphingolipid metabolites, lyso-GalCer and dimethylsphingosine, made it possible to examine their physical response to PS in membranes with lipid characteristics reflective of the plasma membrane cytoplasmic leaflet. The sphingoid bases remained dispersed and mobile. Dimethylsphingosine was remarkably insensitive in terms of orientation and motional order. Since the sugar residue itself of lyso-GalCer is uncharged, the striking conformational changes seen for it seem likely to reflect charge-induced packing effects associated with the amino function closer to the membrane. It is interesting that Perillo et al. [14] emphasised the possibility that (small) packing changes associated with the sphingoid base amino function might be translated into amplified surface properties. Our results certainly support the concept [9,13,14] that charge effects can induce sphingoid base packing changes which have the potential to influence attached extramembranous structures.

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