Investigation of the Calcium-mediated Association between the Carbohydrate Head Groups of Galactosylceramide and Galactosylceramide I3 Sulfate by Electrospray Ionization Mass Spectrometry*

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Calcium has been shown previously to cause aggregation of phosphatidylcholine/cholesterol liposomes containing galactosylceramide (GalCer) with similar liposomes containing cerebroside sulfate (galactosylceramide I3 sulfate) (CBS), suggesting that it mediates a carbohydrate-carbohydrate association between these two glycolipids. In order to determine if such an association occurs, the noncovalent complexes formed on addition of calcium chloride to GalCer and CBS in methanol were examined by positive and negative ion spray mass spectrometry. Monomeric Ca2+ complexes of both lipids were observed. In addition, Ca2+ also caused oligomerization of GalCer. Oligomerization of CBS anion was not seen, but dimers would not have been observed, as they would be neutral. However, Ca2+ caused heterotypic complexation of GalCer and CBS. Although these heterotypic complexes were of low abundance in methanol compared with the other monomeric and homotypic oligomeric positive ions formed at low declustering potentials, the heterotypic dimer [GalCer-CBS-Ca2+]H+ had the greatest stability of all oligomers forming and was the only one to survive at high declustering potentials. Na+ did not cause oligomerization of GalCer in methanol indicating that the complexes of GalCer with Ca2+ are not caused by van der Waals interactions between the lipid moieties. GalCer and CBS are present in high concentrations in myelin. This Ca2+-mediated carbohydrate-carbohydrate interaction, which can bridge apposing bilayers, may be involved in adhesion of the extracellular surfaces of the myelin sheath.

Calcium-mediated interactions between cell surface carbohydrates have recently been implicated as a basis of cell recognition and adhesion and have therefore been the subject of increasing interest (1–8). Carbohydrate-carbohydrate interactions between free sugars and polysaccharides have been known for some time (9–14) and have more recently been investigated among glycolipids in lipid bilayers (15–19). The list of glycolipids that have been shown to participate in carbohydrate-carbohydrate association between these two glycolipids across apposing membrane surfaces might play a role in the formation of the compacted myelin membrane. x-ray crystallography of a number of divalent cation complexes of simple carbohydrates has provided detailed information concerning the structure of these complexes (cf. Refs. 9 and 10 for reviews). NMR and infrared spectroscopy have complemented this information (11, 12). However, the complex-forming properties of calcium with carbohydrates attached to lipid moieties remain largely unexplored. Most of the evidence for complex formation so far comes from liposome aggregation or lipid binding studies using solid phase presentation of the lipids, either bound to a solid support or in liposomes. In addition, Fourier transform-infrared spectroscopy was used to provide information about the structure of the complex of Ca2+ with digalactosyldiacylglycerol and the groups on the carbohydrate that chelate with the divalent cation in a membranous environment in the presence of water (19). The polyvalent nature of presentation in lipid bilayers or on a solid support increases the affinity of the interaction. The difficulty of observing the divalent cation-carbohydrate interactions in solution (especially in aqueous solution) given their weak nature and the solubility characteristics of lipid-bound carbohydrates makes the study of such interactions rather challenging.

Electrospray ionization mass spectrometry (ESI-MS) is a relatively new technique, which can detect the presence of a complex in a solvent in which it is soluble (21–25). The soft ionization conditions employed allow the transfer of complexes present in solution to the gas phase with minimal decomposition (26). ESI-MS has recently been used to detect the divalent cation-mediated complexation of the carbohydrate of some glycolipids, resulting in the homotypic and in some cases heterotypic oligomerization of these lipids in methanol (21). This technique was successful at detecting Ca2+-oligomerization of the Leb oligosaccharide (21), while 1H-NMR failed to detect metal binding or evidence of oligomerization of the free sugar in water (27).

In this paper we present evidence for the noncovalent association among galactosylceramide, Ca2+, and the anion of cerebroside sulfate in methanol solution using electrospray ionization mass spectrometry. Positive ion scans showed that Ca2+-caused homotypic oligomerization of GalCer and that it bound to monomers of CBS anion to form a singly charged positive ion...
ion. Dimers of CBS anions with Ca\(^{2+}\) form a neutral species and are not detected. However, Ca\(^{2+}\) caused heterodimerization of GalCer and CBS anion in methanol. Although the heterotypic complexes were of low abundance compared with the others at low declustering potentials, the heterotypic dimer had greater stability than any other complex.

**EXPERIMENTAL PROCEDURES**

All reagents and solvents used were either analytical grade or high pressure liquid chromatography grade. Calcium chloride (CaCl\(_2\cdot2\)H\(_2\)O) was purchased from Fisher (Fairlawn, N. J). Methanol was from Caledon (Georgetown, Ontario, Canada), stearic acid from Fluka (Switzerland), 1,2-di-O-galactosyldi-sn-glycerol (psychosine) from Sigma, and oxalyl chloride from Aldrich.

Synthesis of Lipids—Galactosylceramide was synthesized from psychosine by reaction with stearoyl chloride (prepared from stearic acid purchased from Fisher, Fairlawn, N. J.). Sphingosine by reaction with stearoyl chloride (prepared from stearic acid purchased from Aldrich.

**RESULTS AND DISCUSSION**

Binding of Galactosylceramide to Ca\(^{2+}\)—The complex-forming property of galactose with Ca\(^{2+}\) in crystalline form has been well documented (30). In the crystal structure, Ca\(^{2+}\) is coordinated to five hydroxyl groups, contributed by three α-D-galactose molecules, and to three water molecules. Since each galactose can provide only one or two hydroxyl groups for Ca\(^{2+}\) binding, single isolated galactose moieties have a low affinity for Ca\(^{2+}\) in aqueous solution. However, the Ca\(^{2+}\)-galactose complex may be more stable in methanol due to the lower tendency of Ca\(^{2+}\) to associate with methanol than water (31). In GalCer, the β-conformation of the sugar and the presence of the ceramide aglycone may also modify the binding to Ca\(^{2+}\). The positive ion ESI mass spectrum of GalCer (monoisotopic mass 727.6) in the presence of excess Ca\(^{2+}\) (Fig. 1) indicates that in addition to the monomeric complex ion of GalCer with Ca\(^{2+}\) ([GalCer-Ca\(^{2+}\)]\(^{+}\)), which is found in high abundance, the lipid forms several oligomers as well, of the general formula [nGalCer-Ca\(^{2+}\)]\(^{+}\). This behavior is similar to that of Le\(^{2+}\)-lactosylceramide (21). A small amount of the Na\(^{+}\) complex is also present at m/z = 750.6.

It is reasonable to assume that the oligomers of GalCer are formed by interaction of the carbohydrate head groups through coordination with Ca\(^{2+}\). As will be discussed later, the relatively higher stabilities of the oligomers, especially the dimer compared with the monomer, lend some support to this suggestion, since the coordination of hydroxyl groups from more than one carbohydrate moiety with the cation has been shown to be the preferred arrangement in the crystal structures of the Ca\(^{2+}\) complexes of many carbohydrates including galactose. It should also be noted that in the presence of Na\(^{+}\), the monomeric ion ([GalCer-Na\(^{+}\)]\(^{+}\)) was the primary ion present in the spectrum with only trace amounts of a dimeric species, [2GalCer-Na\(^{+}\)]\(^{+}\) (not shown). There was no evidence of any higher oligomers containing Na\(^{+}\). As will be seen later, Na\(^{+}\) appears to have a high affinity for GalCer to form the monomeric ion, but even in the absence of Ca\(^{2+}\), its ability to promote oligomerization is negligible. This supports the conclusion that the oligomers of GalCer formed in the presence of Ca\(^{2+}\) for the most part are not due to van der Waals interaction between the ceramide moieties. The negative ion spectrum of GalCer in the presence of excess calcium chloride on the other hand showed predominantly a peak due to a monomeric ion at m/z = 762.4 (not shown), which was identified as [GalCer + Cl\(^{-}\)]\(^{−}\). It persisted at declustering potentials of −80 to −180 V.

Binding of GalactosylceramideI\(^{3}\) Sulfate to Ca\(^{2+}\)—In contrast to GalCer, CBS (monoisotopic mass 807.6) in the presence of excess Ca\(^{2+}\), under the same conditions as above, gives a single adduct with Ca\(^{2+}\) corresponding to the formula [CBS-Ca\(^{2+}\)-H\(^{+}\)]\(^{−}\) (m/z = 846.6) as evident from the positive ion spectrum shown in Fig. 2. It is of lower intensity than that of the complex ions of GalCer. Neutral dimeric species of CBS anion of the type [2CBS-Ca\(^{2+}\)-2H\(^{2+}\)], although possible, would not be detected. The only ion detected in a negative ion scan was [CBS-H\(^{−}\)] in high abundance at m/z 806.4 (not shown). Negatively charged trimers of the type [3CBS-Ca\(^{2+}\)-3H\(^{−}\)] have too high an m/z ratio to be detected by the mass spectrometer used.

Complex Formation between GalCer and CBS in the Presence of Ca\(^{2+}\)—In order to detect Ca\(^{2+}\)-mediated complex formation between GalCer and CBS, solutions containing the two lipids at a concentration of 20 nmol/ml each and Ca\(^{2+}\) at a concentration of 200 nmol/ml in methanol were used. The positive ion ESI mass spectrum of the mixture was acquired under similar conditions as for the individual lipids. Fig. 3 shows a representative spectrum at a declustering potential of 80 V. In addition to the Ca\(^{2+}\)-adducts of the individual lipids as described above, there are hetero-oligomers of the two lipids detectable in the spectrum at m/z = 1170.6 ([2GalCer-CBS-2Ca\(^{2+}\) -2H\(^{2+}\)], 1211.0 ([GalCer-2CBS-2Ca\(^{2+}\)-2H\(^{2+}\)]), and 1574.2...
With an increase in declustering potential, the various Ca\(^{2+}\) adducts of the two lipids disappear. Surprisingly, at 180 V (Fig. 4) the only Ca\(^{2+}\) adducts still remaining are [CBS-Ca\(^{2+}\)-H]\(^+\) (m/z = 846.6), the homodimer of GalCer (m/z = 747.8), and the heterodimer [GalCer-CBS-Ca\(^{2+}\)-H]\(^+\) (m/z = 1574.6). The former two ions are considerably reduced in intensity. As the declustering potential is increased from 50 to 180 V, the first Ca\(^{2+}\) complex of GalCer to disappear from the spectrum is the monomer [GalCer-Ca\(^{2+}\)]\(^+\), and the last to survive in vestigial amounts is the dimer [2GalCer-Ca\(^{2+}\)]\(^+\). This may allow a qualitative comparison of their stabilities to be made, the monomer being the least stable, the dimer the most stable, and other oligomers somewhere in between.

Although almost all of the Ca\(^{2+}\) adducts of GalCer dissociated at a declustering potential of 180 V (Fig. 4), there is a high intensity peak at m/z = 750.6 due to the retention of the Na\(^-\) adduct. The new peaks at 408.2 and 348.2 are fragment ions, as confirmed by parent ion scans, indicating that under the conditions of the experiment significant fragmentation of the lipids occurs.

The ratio of intensity of the heterodimer to that of the CBS monomer increases with increase in declustering potential as shown in Table I. While it is not clear whether there is actually an increase in the abundance of the heterodimer ion with increase in declustering potential, since the intensity of the monomer shows a gradual decrease, the conclusion that the heterodimer [GalCer-CBS-Ca\(^{2+}\)]\(^+\) is the most stable of all the Ca\(^{2+}\) adducts of either lipid, despite its low abundance, seems inescapable.

The low abundance of this complex in methanol contrasts with liposome aggregation studies, which indicated that the Ca\(^{2+}\)-mediated heterotypic aggregation of GalCer with CBS anion was greater than the Ca\(^{2+}\)-mediated homotypic interaction of either. The polyvalent nature of liposomes compared with the monovalent lipids in solution may contribute to the greater heterotypic interaction of liposomes. This difference in behavior may also be due to the relative stability of the Ca\(^{2+}\) adducts of GalCer in methanol solution compared with water due to the lower ability of Ca\(^{2+}\) to chelate methanol compared with water (31). The greater solubility of galactose and other sugars in methanol containing dissolved calcium chloride than in pure methanol, in which they are virtually insoluble, is well documented (32) and also indicates the formation of Ca\(^{2+}\)-galactose complexes in agreement with the ESI-MS results.

CID Analysis of the [GalCer-CBS-Ca\(^{2+}\)-H]\(^+\) ion—Collision-induced decomposition of the heterodimer of GalCer and CBS was accomplished by selecting the ion at m/z = 1574.2 with the mass analyzer and allowing it to collide with argon. The resulting fragments were analyzed in the positive ion mode. Fig. 5
The occurrence of predominantly covalent decomposition in preference to noncovalent decomposition but the ion at m/z 408.4 is its decomposition product. The collisional energy thus appears to be entirely utilized for covalent decomposition in preference to noncovalent decomposition of the complex. The occurrence of predominantly covalent dissociation of complexes under CID conditions has been interpreted as the result of the tightness of the binding of the components (21). Thus in addition to confirming that the complex with m/z = 1574.2 is a heterodimer of GalCer and CBS with Ca\(^{2+}\), the CID experiment also corroborates the finding that this complex is relatively stable, since it undergoes mainly covalent decomposition. However, it is interesting to note that it is the GalCer component of the complex that appears to undergo decomposition while the CBS is unaffected. Thus it is the GalCer component of the complex that appears to undergo decomposition while the CBS is unaffected. The ion at m/z 408.4 is a heterodimer of GalCer and CBS.

Conclusions—In this study we have demonstrated binding of Ca\(^{2+}\) to each of GalCer and the anion of CBS individually. We have also demonstrated the existence of a specific interaction between GalCer and CBS anion mediated by Ca\(^{2+}\) as suggested by the divergent cation-mediated aggregation of phosphatidylcholine/cholesterol liposomes containing GalCer with similar liposomes containing CBS (6, 18). Oligomerization of GalCer, but not of CBS anion, by Ca\(^{2+}\) also occurred in methanol. However, the low degree of aggregation of GalCer-containing liposomes by Ca\(^{2+}\) compared with the greater degree of aggregation of CBS liposomes with GalCer liposomes suggests that in water this homotypic interaction must be much weaker than the heterotypic interaction and/or that the heterotypic interaction is stronger in water than in methanol. The observation of the heterotypic complex [GalCer-CBS-Ca\(^{2+}\)–H]\(^{+}\) in the ESI mass spectrum even under conditions harsh enough to dissociate all other intermolecular clusters points to the specificity of this interaction and stability of the complex.

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REFERENCES

1. Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M., and Hakomori, S. (1989) J. Biol. Chem. 264, 9476–9484
2. Kojima, N., and Hakomori, S. (1989) J. Biol. Chem. 264, 20159–20162
3. Kojima, N., and Hakomori, S. (1991) J. Biol. Chem. 266, 17532–17538
4. Misevic, G. N., and Burger, M. M. (1993) J. Biol. Chem. 268, 4922–4929
5. Dammer, U., Popescu, O., Wagner, P., Anselmetti, D., Guntherodt, H. J., and Misevic, G. N. (1995) Science 267, 1173–1175
6. Hakomori, S. (1991) Pure Appl. Chem. 63, 473–482
7. Kojima, N., Shiota, M., Sadahira, Y., Handa, K., and Hakomori, S. (1992) J. Biol. Chem. 267, 17264–17270
8. Huang, R. T. C. (1978) Nature 276, 624–626
9. Cook, W. J., and Bugg, C. E. (1977) in Metal Ligand Interactions in Organic Chemistry and Biochemistry, Part 2 (Pullman, B., and Goldblum, N., eds) pp. 231–256, Reidel Publishing Co., Dordrecht, Holland
10. Whitfield, D. M., Stojkowsky, S., and Sarkar, B. (1993) Coord. Chem. Rev. 122, 171–225
11. Angyal, S. J. (1972) Aust. J. Chem. 25, 1857–1866
12. Angyal, S. J., and Davies, K. P. (1973) Chem. Commun. 500–501
13. Varela, B. S., and Albersheim, P. (1974) Plant Physiol. 54, 105–108
14. Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., and Thom, D. (1973) FEBS Lett. 32, 195–198
15. Webb, M. S., Tilcock, C. P. S., and Green, B. R. (1988) Biochim. Biophys. Acta 938, 323–333
16. Gupta, D., Arango, R., Sharon, N., and Brewer, C. F. (1994) Biochemistry 33, 2503–2508
17. Brewer, G. J., and Matinyan, N. (1992) Biochemistry 31, 1816–1820
18. Stewart, R. J., and Boggs, J. M. (1993) Biochemistry 32, 10666–10674
19. Mesnich, M. A., and Fragata, M. (1993) Eur. J. Biochem. 212, 249–258
20. Norton, W. T. (1977) in Myelin (Morel, P., ed) pp. 161–199, Plenum Press, New York
21. Saidak, G., Ichikawa, Y., Caulfield, T. J., Munce, B. W., Wong, C. D., and Nicolau, K. C. (1993) J. Am. Chem. Soc. 115, 2877–2881
22. Smith, R. D., Light-Wahl, K. J., Winger, B. E., and Loo, J. A. (1992) Org. Mass Spectrom. 27, 811–821
23. Saidak, G., Zheng, Z., Rhamphal, J. Y., Ichikawa, Y., Nicolau, K. C., Gaeta, F. C. A., Chatman, K. S., and Wong, C-H. (1994) Bioorg. Med. Chem. Lett. 4, 2863–2866
24. Camilleri, P., Haskins, N. J., and Saunders, M. R. (1993) Rapid Commun. Mass Spectrom. 7, 949–952
25. Camilleri, P., Haskins, N. J., and Howlett, D. R. (1994) FEBS Lett. 341, 286–288
26. Smith, R. D., and Light-Wahl, K. J. (1993) Biol. Mass Spectrom. 22, 493–501
27. Wornald, M. R., Edge, C. J., and Dwek, R. A. (1991) Biochim. Biophys. Acta 1080, 1214–1221
28. Kopaczky, K. C., and Radin, N. S. (1965) J. Lipid Res. 6, 140–145
29. Koshy, K. M., and Boggs, J. M. (1983) Chem. Phys. Lipids 34, 41–53
30. Cook, W. J., and Bugg, C. E. (1973) J. Amer. Chem. Soc. 95, 6442–6446
31. Rendelman, J. A., Jr. (1966) Adv. Carbohydr. Chem. 21, 209–271
32. Domov, K. B., and Freund, E. H. (1960) J. Dairy Sci. 43, 1216–1223
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