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PROPERTIES OF IONIC TRANSPORT THROUGH PHOSPHOLIPID-GLYCOLIPID ARTIFICIAL BILAYERS

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The ionic transport properties of dioleoylphosphatidylcholine (DOPC) membranes containing various sphingolipids were studied. Particular attention was paid to membranes formed from β-D-glucosylceramide (GlcCer) and DOPC. They showed a marked increase in ionic permeability (up to a factor 30 with respect to pure DOPC membranes), slight cation selectivity and almost linear behaviour of the current-voltage characteristic. Bilayers containing GlcCer showed a typical conductance decrease upon increasing the temperature. We suggest the formation of clusters containing GlcCer molecules in a solid-crystalline phase. The conductance increase might be due to ionic pathways through disordered boundary regions. An increase in the mechanical breakdown potential was observed in all membranes which contain sphingolipids.

Introduction

Evidence has recently been presented concerning the fundamental role played by glycosphingolipids and glycoproteins in many cell-surface phenomena. Glycolipids are involved in cell-cell interaction [1], in cell growth and oncogenic transformations [2,3], in recognition of external ligands [4–8], in antigenic reactions [1,9]. A modification of glycolipid cellular content characterizes many inborn pathological conditions, such as Nieman-Pick's, Krabbe's, Fabbry's, Tay-Sach's and Gaucher's diseases [10].

The complexity of oligosaccharide chains and the aggregation of glycolipids influence membrane structure and fluidity markedly [3,11,12]. Proteins, and mainly glycoproteins, are involved in such a segregation process.

This paper describes the behaviour of black lipid membranes made from various mixtures of sphingolipids and dioleoylphosphatidylcholine. Particular attention is devoted to effects induced by *β*-D-glucosylceramide, the abnormally high concentration of which is a characteristic of spleen and liver lysosomes or brain cells of patients affected by Gaucher's disease [10]. It possibly derives from an incomplete degradation of gangliosides [13]. We suggest that the lateral phase separation of a two-component lipid system can also be detected by its effect on ionic transport properties.

Materials and Methods

DOPC, β-D-glucosylceramide, β-D-galactosylceramide (GalCer) and ceramide were obtained

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Abbreviations: GlcCer, glucosylceramide; GalCer, galactosylceramide; DOPC, dioleoylphosphatidylcholine.

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TABLE I

SPHINGOLIPID FATTY ACID DISTRIBUTION

Primarily fatty acid, R, composition of used sphingolipids. The distribution was determined by gas-chromatographic analysis. All compounds were characterized by 4-sphingenine ($C_{18:1}$) at a percentage of 99%. A good correlation exists between the ceramides and the hydroxyglycosylceramides from which were prepared. As concerns these last compounds, about 10% and 20%, respectively, of fatty acid residues were not identified, but we have indications that they belong to the 'hydroxy' category.

R	GlcCer	Hydroxy-GalCer	Non-hydroxy GalCer	Ceramide
⩾ 25:0			2.6	
⩾ 25:1			15.0	
24:0	23.2	55.8	14.6	57.4
24:1	6.1		43.5	
23:0	8.0		4.7	
22:0	31.7		4.7	
20:0	7.2			
18:0	7.4	34.2	11.2	22.5
16:0	16.1			

from Sigma Chemical Company, St. Louis, MO, U.S.A. GlcCer was prepared from the spleen of patients affected by Gaucher's disease, while GalCer and ceramide were extracted from bovine brain. GalCer was obtained separately in two forms: primarily non-hydroxy fatty acids (upper spot GalCer) and primarily hydroxy fatty acids (lower spot GalCer). The fatty acid composition of sphingolipids, gas-chromatographically determined, is reported in Table I. Our results are in good agreement with data from Marinetti et al. [14] on Gaucher spleen glucosylceramides.

In some control experiments we used GlcCer (Gaucher-affected spleen) purchased from Supelco Inc., Bellefonte, PA, U.S.A.

All phospholipid and sphingolipid samples gave single spots on thin-layer chromatography plates (Merck silica gel H in chloroform/methanol/water (65 : 35 : 5, v/v) and they were therefore used without any further purification.

Black lipid membranes were formed according to the brush technique of Mueller et al. [15]. Stable membranes were formed from different mixtures of sphingolipids and DOPC, with the mole ratio of sphingolipid ranging from 0 to 0.5. The total lipid amount in n-decane was kept constant at 15 mg/ml. Mixtures of sphingolipids and DOPC were prepared in chloroform/methanol (2 : 1, v/v), evaporated under nitrogen and then dissolved in n-decane. Gentle heating to about 40°C was required to obtain clear solutions when mixtures containing a higher proportion of sphingolipid were used.

The experimental arrangement includes two Teflon compartments separated by a Teflon septum with a 0.7 mm hole across which the lipid bilayers are formed. If not otherwise indicated, the salt bathing solution was identical on both sides. The same Ag/AgCl electrodes were used to apply external voltages and to record the current flowing through the membrane. All experiments were performed in voltage clamp. The temperature was kept constant at 24 ± 0.5°C by water circulating from an external thermostatically controlled bath.

Results

Ion-transport properties of black lipid membranes containing glycosphingolipids have been studied by various authors [16–19]. With the aim of correlating some functional properties with membrane structure and composition, we analyzed the permeability properties of black lipid membranes made from various percentages of DOPC and sphingolipids.

We were not able to obtain stable membranes from pure sphingolipids dissolved in n-decane, while black lipid membranes made of pure DOPC were checked for stable low conductance over several hours: g_0 (DOPC) $= (4 \pm 1) \cdot 10^{-8}\ \Omega^{-1} \cdot \mathrm{cm}^{-2}$.

In Fig. 1 is reported on a semilogarithmic scale the zero-current stationary conductance of black lipid membranes formed from various mixtures of monoglycosylceramides and DOPC. The ionic conductance increases by increasing the glycolipid:DOPC mole ratio, c. Marked permeability modifications can be observed for GlcCer:DOPC membranes, while GalCer:DOPC mixtures showed lower conductance values (in the order of pure DOPC membranes). For $c > 0$ experimental points in Fig. 1 were fitted by straight lines in the semilogarithmic scale. This means that each stationary specific conductance can be expressed as a function of the glycolipid:DOPC mole ratio by a phenomenological equation of the type:

$$g_0 = \bar{g}\, e^{\alpha c} \qquad (1)$$

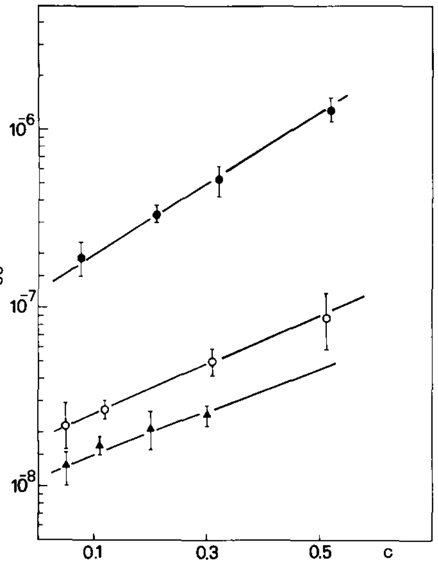

Fig. 1. Ionic steady-state conductance, g, vs. the glycolipid:DOPC mole ratio, c. ●, GlcCer:DOPC; ○, hydroxy GalCer:DOPC; ▲, non-hydroxy GalCer:DOPC. Each point is the mean value obtained from at least ten different experiments. The mean conductance of pure DOPC membranes was $g_0 = (4 \pm 1) \cdot 10^{-8}\ \Omega^{-1} \cdot cm^{-2}$. 100 mM NaCl, 1 mM $CaCl_2$, pH 7. Experiments performed at 24±0.5°C.

where α and $\bar{g}$ are parameters which depend on the nature of the glycolipid but are independent of its concentration.

For glycolipid mole ratios higher than 0.5, the lipid bilayers showed fluctuations in the ionic conductance and lower mechanical stability.

Membranes made from ceramide/DOPC mixtures did not show significant dependence of ionic transport properties as a function of the mole ratio. The conductance values, $g_0\ (\text{cer}) = 10^{-8}\ \Omega^{-1} \cdot cm^{-2}$, seem to be a little lower with respect to the conductance of pure DOPC membranes.

In control experiments we used β-D-glucosylceramide (Gaucher-diseased spleen) purchased from Supelco, and we found that conductance values were in good quantitative agreement with the experimental points reported in Fig. 1. Together with thin-layer chromatography, this fact supports the view that our results are not due to impurities of the glycolipids, which are expected to depend on the extraction method of GlcCer.

We verified that a low concentration of calcium has no effect on membrane permeability properties, by observing that symmetrical addition of 1 mM $CaCl_2$ did not affect the conductance of GlcCer:DOPC membranes formed in 100 mM NaCl solutions at pH 7. Therefore, we performed selectivity measurements in absence of calcium. The relative permeability of the membrane for Na^+ and Cl^- was tested as shown in Fig. 2. Data refer to a membrane formed from GlcCer and DOPC at a mole ratio of 0.11. Experimental values were best fitted by the Goldman-Hodgkin-Katz equation [20,21]:

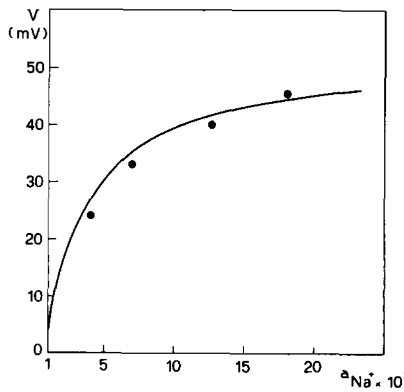

Fig. 2. Steady-state potential as a function of increasing salt activity on one side of the membrane. Small volumes of 4 M NaCl were added successively. The starting concentration of NaCl was 10 mM. GlcCer:DOPC mole ratio, 0.11. The curve is drawn according to the Goldman-Hodgkin-Katz equation. $P_{Na^+}/P_{Cl^-} = 8$.

$$\Delta V = V_i - V_o = \frac{RT}{F} \ln \frac{(P_{Na^+}/P_{Cl^-})a^o_{Na^+} + a^i_{Cl^-}}{(P_{Na^+}/P_{Cl^-})a^i_{Na^+} + a^o_{Cl^-}} \qquad (2)$$

which correlates the potential differences, ΔV, developed between the two sides of the membrane to the activities, a_{Na^+} and a_{Cl^-}, of the *trans* side (labelled i) and the *cis* side (labelled o) of the cell. A permeability ratio (P_{Na^+}/P_{Cl^-}) of 8 was obtained.

This result is in accordance with observations of other authors who measured small cationic selectivity in GalCer:phospholipid membranes [18,19]. It may depend on the surface charge of our bilayers, arising from the orientation of the various dipole moments existing at the membrane surface and due to the choline group, the ester linkage or the glucose terminal [22].

We performed experiments in which the specific conductance was measured as a function of the temperature. A typical trend is reported in Fig. 3; (a) for a GlcCer:DOPC membrane (mole ratio = 0.30) and (b) for a DOPC membrane. The specific conductance (referred to the conductance at 24°C) of pure DOPC membranes increases upon increasing the temperature.

Conversely, when glucosylceramide was present in a DOPC bilayer, the conductance decreased by about one-third for a 25 K temperature increase. The GlcCer:DOPC membrane showed a higher conductance when the temperature reverted from about 50°C to 24°C. It may be that a higher temperature induces an increase in the GlcCer concentration in the membrane phase and therefore a higher conductance when the system reverts to lower temperatures.

The behaviour of GlcCer:DOPC membranes as a function of the applied potential was studied by performing current-voltage experiments. As reported in Fig. 4, the *I*-*V* curve is symmetric and presents a slight deviation from linearity only at higher potentials.

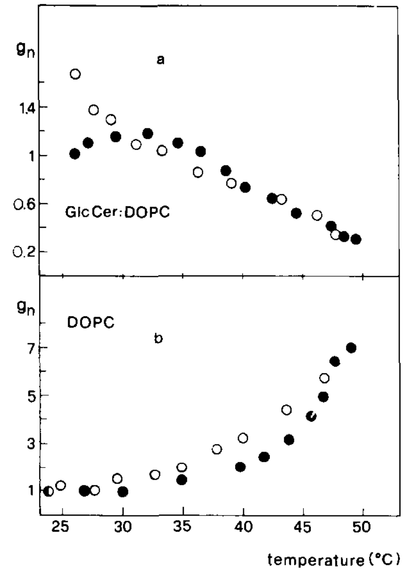

Fig. 3. Normalized steady-state membrane conductance $g_n = g_0(T)/g_0(24°C)$ reported as a function of the temperature, *T*, for (a) a GlcCer:DOPC membrane (mole ratio, 0.3), (b) a pure DOPC membrane. ●, Increasing *T*; ○, decreasing *T*. $\Delta T/\Delta t = 1$ deg./min. Initial temperature: $T = 24°C$. 100 mM NaCl, 1 mM $CaCl_2$; pH 7.

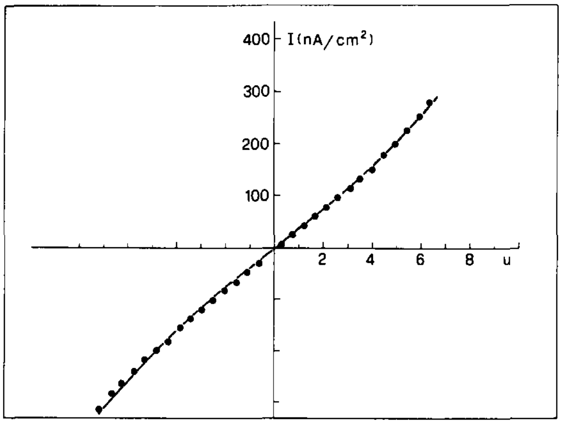

Fig. 4. Current-voltage relationship for a black lipid membrane made from GlcCer:DOPC (mole ratio, 0.3). $u = VF/RT$ = reduced potential. The experiment was performed at $\Delta V/\Delta t = 2$ mV/s. Only discrete current values are reported, in order to show more clearly the best fit with Eqn. 3. 100 mM NaCl, 1 mM $CaCl_2$; pH 7.

As concerns membrane stability, bilayers containing glucosylceramide molecules were stable over many hours and the mechanical rupture upon applied voltages was higher than that found in pure DOPC membranes. Moreover, the presence of the other sphingolipids also enhanced mechani-

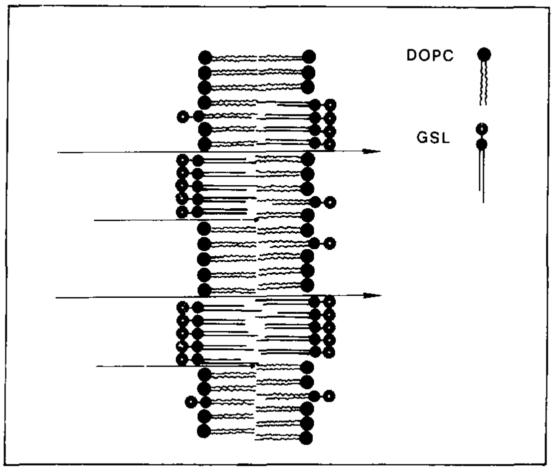

Fig. 5. Tentative schematic model of lateral phase separation in a black lipid membrane made from glycosylceramide and dioleoylphosphatidylcholine. Glycolipid monomers are in a fluid configuration. Clusters correspond to solid domains. Poor interactions among different polar heads as well as hydrophobic tails determine disordered boundary regions.

cal and electrical membrane stability. Experiments were performed at a sphingolipid:DOPC mole ratio of 0.30, (100 mM NaCl, 1 mM $CaCl_2$; pH 7). The applied voltage was slowly increased until film rupture occurred. We obtained the following values of the breakdown voltage referred to the mean voltage (210 ± 10 mV) at which the rupture of pure DOPC bilayers occurs. Glucosylceramide, 1.48 ± 0.08; hydroxy galactosylceramide, 1.20 ± 0.07; non-hydroxy galactosylceramide: 1.15 ± 0.15; ceramide: 1.32 ± 0.26. Errors were evaluated by using the Student *t* distribution with a confidence coefficient, $P = 99\%$.

Discussion

It is well known that many molecular species are subjected to clustering phenomena in native membranes. These spatial organizations of various components could control some functions of the cell surface, for example, signal amplification and transport properties [23–25]. It is also generally accepted that two-dimensional lipid mixtures can give rise to lateral phase separations of the components [26–28]. The existence of fluid and crystalline domains was shown by using different techniques such as electron microscopy [27], differential scanning calorimetry [29], electron spin resonance [30], calorimetric heat capacity curves [28] and X-ray diagrams of liquid binary alloys [31].

Calorimetric studies have shown that lipid binary mixtures, characterized by very different transition temperatures, present a decrease and a broadened melting pattern upon the lateral phase separation of the two compounds [16,32]. Clusters or solid microdomains of the compound melting at higher temperature may occur, depending on different parameters such as temperature of the system and mole ratio of the two substances. Clowes et al. [16] have reported that hydrated ox-brain cerebrosides have a phase transition at 55°C. It falls to 20°C at GalCer:phosphatidylcholine mole ratio of 1 : 1. We performed preliminary differential scanning calorimetry experiments and found a gel-to-liquid-crystalline transition of pure hydrated glucosylceramide (10 mg/1 ml water solution) at about 80°C. Therefore, we suggest that GlcCer:DOPC mixtures should present mainly, at room temperature, a partial gel configuration in which fluid (phospholipid and glycolipid) monomeric distribution will contain solid phase clusters.

As occurs in the range of the phase transition of pure lipids [31,33–35] and also in multicomponent systems, correlations have been reported between the physical state of membranes and their transport properties [30,36–39]. Furthermore, various authors have suggested a modification of the ionic energy barrier due to the penetration and interaction of water molecules within the lipid bilayers [40–42]. In the case of solid microdomains dispersed in a fluid medium, the boundary effect will favour a more pronounced water penetration in the membrane hydrophobic core [43].

These considerations give rise to a tentative interpretation of our results in terms of a simple model (Fig. 5) based on the hypothesis that lateral phase separation of glycolipids determines an increase of the ion permeability through disordered boundary regions. As far as ceramide is concerned, it presents only hydroxy fatty acids; moreover, it does not have any glycosyl terminal. Therefore, it is not surprising that ceramide behaves in a manner different from glycolipids. It probably confers higher viscosity to the membrane, but no clusters occur.

It seems quite improbable that high permeability pathways exist inside microscopic solid domains. In fact, the mean area occupied by molecules in a solid crystalline configuration is lower with respect to the area occupied by the same molecules in a fluid state [43]. The close interaction between aggregated molecules determines lower mobility and higher ionic diffusion coefficients inside solid clusters [31]. On the basis of these considerations, various authors generally ascribe to boundary regions the ability to form ionic pathways [31,40].

In any case, the results of experiments on the effects of temperature support the cluster hypothesis, rather than that of effects dependent on the glucosylceramide monomeric distribution. A slow and broad melting of clusters involves a diminution in the disordered areas and therefore a decrease in the membrane permeability.

According to our tentative interpretation, this result would imply that the number of glucosylceramide clusters decreases upon an increase in the temperature, due to the higher solubility of GlcCer in DOPC. This effect would dominate those induced by the higher fluidity of the bilayer and the increase of the ionic partition coefficient. In fact, the conductance behaviour observed for DOPC is consistent with a decrease in the bilayer viscosity and with an increase in the partition coefficient of the ion between the membrane phase and aqueous solution. Therefore, it must be considered that the conductance decrease induced by the presence of glucosylceramide molecules is even greater than the factor 0.3 observed in Fig. 3a.

Under our conditions, the transition from a partial gel-crystalline array to a liquid-crystalline structure is a broad phenomenon, as it would be when mixtures of lipids with very different transition temperatures are used. Under the hypothesis that ionic permeability modifications depend on a supersaturated concentration of glycolipids in the phospholipid medium, Eqn. 1 is no longer valid in the limit $c \rightarrow 0$, i.e., in the limit of solubility of glycosphingolipids in DOPC.

As far as the current-voltage characteristic is concerned, ion injection, field dissociation (or the Wien effect) and the charge image effect may be responsible for the deviation from linear behaviour [45]. Nevertheless, the injection of ions into the membrane is significant at low ionic strength, while, as concerns the Wien effect, there is no evidence for the existence of high interfacial barriers between membrane and water solution. Consequently, we suggest an interpretation of the observed overlinearity in terms of image forces and dipole potentials which induce a trapezoid-shaped ionic energy barrier [45,46]. Under the following hypotheses: (1) symmetric membrane; (2) constant field approximation, (3) Boltzman distribution of the permeating ions between the bulk solution and the corner of the membrane energy barrier, the following equation can easily be derived [46,47]:

$$I = \frac{ku(e^{u} - 1)}{e^{u(1-n)} - e^{nu}} \qquad (3)$$

where I is current density; $u = VF/RT$ = reduced potential (F, Faraday constant; R, gas constant, T, absolute temperature); $k = g_0 RT/F$ (g_0 = zero current specific conductance), and n is the fraction of the symmetric displacement of the energy barrier.

The best fit of the data in Fig. 4 gave the values for the parameters k and n: $k = 33$ nA/cm; $n = 4.2 \cdot 10^{-2}$. This represents a specific zero-current conductance, g, of $8.5 \cdot 10^{-7}\ \Omega^{-1} \cdot \mathrm{cm}^{-2}$ and a trapezoidal displacement in the order of 5% of the total thickness of the membrane.

The absence of any observable change in the ionic permeability properties upon addition of ceramide to DOPC argues against a correlation between higher ionic transport and higher mechanical stability to external applied voltages. In fact, GlcCer:DOPC membranes exhibited a mechanical rupture at the highest voltage, even if a slight increase was observed also when black lipid membranes containing galactosylceramides or ceramides were used. Our results are in accordance with the results of experiments performed by Clowes et al. [16] on lipid bilayers formed from galactosylceramide/phosphatidylcholine mixtures.

Benz et al. [48] have suggested that a lateral shift of solvent may be responsible of the mechanical rupture of the membrane, the electromechanical compression playing no relevant role. Since it has been demonstrated that the presence of sphingolipids confers higher rigidity to glyceride bilayers [8], the higher mechanical stability of black lipid membranes containing sphingolipids might be ascribed to the higher viscosity of the film. In this hypothesis, the lateral squeezing of the solvent could have a lower destabilizing effect.

A different explanation may reside in the hypothesis that membranes containing sphingolipids present a higher mean thickness due to the long acyl chains which characterize the hydrophobic backbone.

Conclusions

Due to the difference in the fatty acid composition of sphingolipids, we have so far no clear insight as to the role played by the saccharide terminal and hydrophobic chains, respectively. Even though it has been pointed out that in binary mixtures non-ideal mixing of diacyl amphiphiles occurs when the chain length or the degree of saturation of the two components is very different [32,49,50], an important role played by the sugars should be considered. In fact, it is well known that the melting of phospholipids involves the hydrocarbon tails as well as the polar head groups [51–53]. Moreover, it has been demonstrated that membrane glycosphingolipids have a tendency to organize themselves into clusters [8,11,54–56]. This cooperative interaction depends on the structure of the oligosaccharide chains and is particularly evident in the case of gangliosides [12,30]. Concerning β-D-glucosylceramide the distance (4.85 Å) between the hydroxyl oxygens attached to the chiral carbon atoms 2 and 4 of the β-glucose seems to fit very well into the real ice lattice of water [57]. This behaviour could confer higher order to hydrated glucosylceramide molecules with respect to other sphingolipids. Moreover, the fact that conductance increases were not observed in the presence of ceramides may support the hypothesis that glycosyl terminals influence markedly the structure and the organization of the membrane.

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