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Effect of sulfatide and gangliosides on phospholipase C and phospholipase A_2 activity. A monolayer study

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The effect of sulfatide and gangliosides G_{M1}, G_{D1a} and G_{T1b} on the activity of phospholipase C from *Clostridium perfringens* on dilauroylphosphatidylcholine and of porcine pancreatic phospholipase A_2 on dilauroylphosphatidic acid was studied in lipid monolayers containing different proportions of glycolipids under zero-order kinetics at various constant surface pressures. The presence of sulfatide in the monolayer increases the activity of phospholipase C at high surface pressures. Gangliosides shift the cut-off pressure to lower values and inhibit the action of phospholipase C. In mixed monolayers with dilauroylphosphatidic acid, sulfatide at a molar fraction of 0.5 increases the activity of phospholipase A_2 at surface pressures below 18 mN/m and shows an inhibitory effect at higher pressures. Ganglioside G_{M1} at a molar fraction of 0.25 completely inhibits the enzyme above 20 mN/m and markedly reduces its activity at lower pressures. Gangliosides G_{D1a} and G_{T1b} abolish the enzyme activity at all pressures at molar fractions of 0.25 and 0.15, respectively. The modified velocity of the enzymatic reaction in the presence of glycosphingolipids is not due to an irreversible alteration of the catalytic activity.

Introduction

The activation of the endogenous forms of phospholipase C (PLC) and phospholipase A_2 (PLA_2) is thought to mediate signal transduction across membranes and it has been related to a number of cellular events [1–3]. Apart from the physiological importance of these effects, there has been continuous interest in the extreme dependence of the enzyme activities on the composition and intermolecular organization of the interface [4–6]. PLC and PLA_2 from different sources exhibit interfacial activation but lower specific activity toward unilamellar phosphatidylcholine vesicles compared to phosphatidylcholine-detergent mixed micelles [7]. The activation of PLC by Ca^{2+} [5] is analogous to that for PLA_2 [8]. Either PLC or PLA_2 require a certain amount of hydrophobic interaction with the lipid chains for binding to the interface [9].

Gangliosides and other glycosphingolipids (GSLs) have been implicated in the regulation of membrane-mediated phenomena. They have marked effects on the surface organization, thermodynamics and topology [10,11]. These relate to the modulation of protein receptors, ion channels and protein kinases [12,13]. We have recently found that several GSLs including gangliosides modulate dlPC hydrolysis by PLA_2 in lipid monolayers [14]. In this work we have studied the effects of Sulf and gangliosides G_{M1}, G_{D1a} and G_{T1b} on the hydrolysis of dlPC by PLC (*Clostridium perfringens*) and on the activity of PLA_2 towards the negatively charged substrate dlPA.

Materials and Methods

dlPC, dlPA, PLA_2 from porcine pancreas and PLC from *Clostridium perfringens* were from Sigma Chem.

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Abbreviations: dlPC (dilauroylphosphatidylcholine), didodecanoyl-*sn*-glycero-3-phosphorylcholine; dlPA (dilauroylphosphatidic acid), didodecanoyl-*sn*-glycerol-3-phosphate; PLC,phospholipase C; PLA_2, phospholipase A_2; Cer (ceramide), *N*-acylsphingosine; Sulf (sulfatide), Gal(3-sulfate)β1-1′Cer; G_{M1} (monosialoganglioside): Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1′Cer; G_{D1a} (disialoganglioside), NeuAcα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc)β1-4Glcβ1-1′Cer; G_{T1b} (trisialoganglioside), NeuAcα2-3Galβ1-3GalNAcβ1-4Gal(3-2αNeuAc8-2αNeuAc)β1-4Glcβ1-1′Cer.

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Co. (St. Louis, MO, U.S.A.). Over 95% of the protein ran as a single band on SDS-PAGE. No changes of surface tension or surface potential occurred in lipid-free interfaces when the enzymes were injected into the subphase at a concentration 10-fold higher than that routinely used and the surface area was reduced to 10% of the initial value. The purification and sources of GSLs, the technique, equipment, calculations and reproducibility for preparing and measuring the monolayer properties were reported in detail previously [14]. PLA_2 activities were measured in a 'zero order' trough coupled to a surface barostat. The values for the enzyme velocity were normalized as recently described [14]. The normalization procedure must be employed in order to report true comparative activities in films of different proportions and with different mean molecular areas at the same pressure. This is in order to account for different dilutions of substrate by the non-substrate lipids. In addition, any deviations from the ideal behavior for mean molecular area due to GSLs–phospholipid interactions [15] must also be considered [14]. PLC activity was measured as reported by Moreau et al. [16] using a two-step reaction system in which an excess of lipase is used to hydrolyze the insoluble 1,2-diacylglycerol to free fatty acid and 2-monoacylglycerol. For short-chain phospholipids these products are immediately desorbed from the interface. Conditions of 'zero-order' kinetics are maintained by continuously supplying substrate to the surface of the reaction compartment from a monolayer reservoir [14]. The constancy of composition on the reaction compartment is further controlled by continuously monitoring the electrical potential across the interface. This parameter is extremely sensitive to the monolayer organization and, therefore, to its composition [14,15,17–19]; any inhomogeneous film losses or variations in the lipid and protein proportions in the monolayers can be precisely controlled. Gangliosides or phospholipids do not desorb from the mixed films into the subphase; the surface pressure- and surface potential-area isotherms were reproducible after several compression and decompression cycles [15,17].

Results and Discussion

Effect of GSLs on dlPC hydrolysis by phospholipase C

The presence of Sulf in mixed monolayers with dlPC induces an increase of the activity of PLC, especially at high surface pressures. The activation also occurs at low surface pressures when the molar fraction of Sulf in the mixture is above 0.25 (Fig. 1). The variations of the enzyme velocity of PLC with the surface pressure occurs with similar lag-times and surface potential changes for monolayers of pure dlPC or for mixed films with Sulf (Table I). Both parameters are related to the interfacial adsorption (and/or penetration) of the enzyme [14]. The surface potential remains constant after the initial enzyme-monolayer interaction and throughout the zero-order kinetics [14]. In addition, this change is similar for the different films (Table I). This indicates that the monolayer lipid-protein composition during the course of the enzyme action must remain, on average, the same. Thus, the specific activity of the enzyme is increased in the film with Sulf, the effect is similar to that found for PLA_2 [14].

The presence of gangliosides G_{M1}, G_{D1a} and G_{T1b} in the film inhibits PLC activity and shifts the cut-off pressure to lower values (Fig. 2). This effect is also

TABLE I

Kinetic parameters and surface potential changes during PLC activity

Bulk enzyme concentration = 0.025 μg/ml. PL, phospholipid.

Film component	Molar fraction of PL	Surface pressure ($mN \cdot m^{-1}$)	Enzyme velocity ($\times 10^{-14}$) (molecules · $cm^{-2} \cdot min^{-1}$)	Lag-time (min)	Difference in surface potential [a] (mV)
dlPC	1	7	1.70	4.0	−40
	1	15	2.72	9.5	−30
dlPC : Sulf	0.5	15	3.53	9.0	−35
dlPC : G_{M1}	0.5	7	1.75	4.5	−45
dlPC : G_{M1}	0.5	10	0.00		−25
dlPC : G_{D1a}	0.5	5	0.78	4.5	−35
dlPC : G_{D1a}	0.5	7	0.00		−30
dlPC : G_{T1b}	0.75	5	1.10	4.0	−35
dlPC : G_{T1b}	0.75	7	0.00		−30

[a] The figures correspond to the constant value obtained after injecting the enzyme into the subphase, beyond the lag-time and throughout the zero-order kinetic regime. The difference is taken with respect to the initial value of surface potential of the lipid monolayer on the enzyme-free subphase at the particular constant surface pressure used.

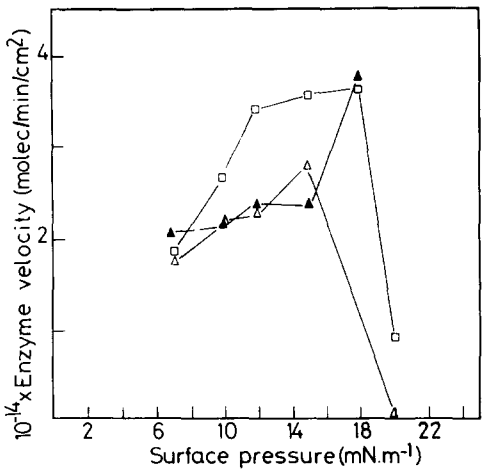

Fig. 1. Effect of Sulf on PLC activity. Normalized enzyme velocity as a function of surface pressure for monolayers of pure dlPC (△) or dlPC with a molar fraction of Sulf of 0.25 (▲) and 0.50 (□). The subphase contained a final concentration of 2.5 nM pancreatic lipase, 25 nM colipase and 2 nM PLC. Buffer: Tris-HCl 20 mM, NaCl 0.15 M, $CaCl_2$ 5 mM, $ZnSO_4$ 0.1 mM, pH 7.2, 30 °C.

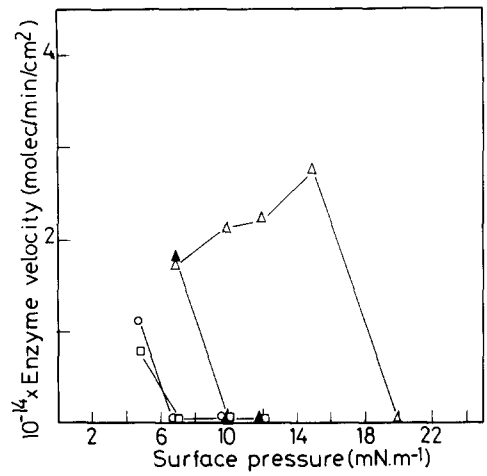

Fig. 2. Effect of gangliosides on PLC activity. Normalized enzyme velocity as a function of surface pressure for monolayers of pure dlPC (△) or dlPC with a molar fraction of 0.50 of G_{M1} (▲) or G_{D1a} (□) or 0.25 of G_{T1b} (○). The other conditions were as in Fig. 1.

similar to that found for PLA_2 [14]. However, a higher proportion of gangliosides in the film is required for a similar inhibition of PLC (see Tables I and II). Similar to PLA_2, the inhibition of PLC occurs at lower molar fractions of gangliosides when their oligosaccharide chain is more complex (Fig. 2).

Effect of GSLs on the hydrolysis of dlPA phospholipase A_2

Anionic phospholipids are better substrates for PLA_2 than neutral phospholipids [20–24]. We have also found this effect with dlPA (Fig. 3). For dlPC, the optimum surface pressure for enzymatic activity is at 12 mN · m^{-1}. Practically no activity is found at 20 mN · m^{-1} and the pressure cut-off point is about 22 mN · m^{-1} [14]. By contrast, the PLA_2 activity against dlPA increases dramatically above 18 mN · m^{-1}. The enzyme velocity at 22 mN · m^{-1} is about 3-times higher than that observed for dlPC (Fig. 3 and Table II). Qualitatively, the cut-off point is above 24 mN · m^{-1}. Unfortunately, in the presence of the enzyme, the films of dlPA become unstable above 24 mN · m^{-1} and the enzyme activity could not be measured at higher pres-

TABLE II

Kinetic parameters and surface potential changes during PLA_2 activity

Bulk enzyme concentration = 0.025 μg/ml. PL, phospholipid.

Film component	Molar fraction of PL	Surface pressure (mN · m^{-1})	Enzyme velocity (×10^{-14}) (molecules · cm^{-2} · min^{-1})	Lag-time (min)	Difference in surface potential [a] (mV)
dlPC	1	15	2.55	5.7	−30
dlPA	1	15	2.73	1.7	−15
dlPC	1	22	0.00		0
dlPA	1	22	8.71	1.5	−15
dlPC : Sulf	0.5	15	3.82	3.1	−30
dlPA : Sulf	0.5	15	4.64	1.5	−15
dlPC : G_{M1}	0.8	15	0.64	3.2	−25
dlPA : G_{M1}	0.9	15	1.85	2.0	−15
dlPC : G_{M1}	0.8	18	0.00		−20
dlPA : G_{M1}	0.9	18	0.00		−10

[a] The figures correspond to the constant value obtained after injecting the enzyme into the subphase, beyond the lag-time and throughout the zero-order kinetic regime. The difference is taken with respect to the initial value of surface potential of the lipid monolayer on the enzyme-free subphase at the particular constant surface pressure used.

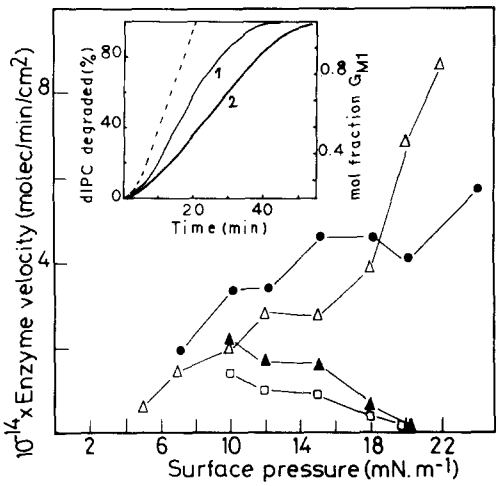

Fig. 3. Effect of ganglioside G_{M1} and Sulf on PLA_2 activity. Normalized enzyme velocity as a function of surface pressure for monolayers of pure dlPA (△) or dlPA with a molar fraction of G_{M1} of 0.10 (▲) and 0.25 (□) or Sulf of 0.50 (●). PLA_2 0.025 μg/ml (final concn.) Buffer: Tris-HCl 10 mM, $CaCl_2$ 20 mM, NaCl 100 mM, pH 8.0, 30°C. Inset: Effect of G_{M1} on the extent of dlPC degradation. Percentage of dlPC degraded as a function of time after injecting at time zero (1) 0.050 μg/ml or (2) 0.025 μg/ml of enzyme under a monolayer of dlPC/G_{M1} (2:1, initial molar ratio) at 10 mN·m^{-1}. The right ordinate gives the molar fraction of G_{M1} in the film as dlPC degradation proceeds. The dashed line shows the degradation of pure dlPC at the same pressure (zero-order kinetics is obtained since no variation of composition occurs).

sures. Contrary to dlPC, dlPA exhibits a two-dimensional phase transition at 20–22 mN · m^{-1} on subphases containing Ca^{2+} [15]. The abrupt increase in PLA_2 activity in this pressure range may be due to packing defects and increased fluctuations in the transition region. These enhance PLA_2 activity [25,26].

The presence of Sulf in the monolayer induces considerable increase of enzyme velocity below 18 mN · m^{-1} but has an inhibitory effect at higher pressures (Fig. 3). In mixed films of Sulf-dlPA, the mean molecular area and average surface potential per molecule are increased with respect to an ideal film [15]. The positive deviations of the surface potential per molecule could result from a more protonated interface [27,28] due to the additional negative charges provided by Sulf. The pK (approx. 2) of this lipid is much lower than the second pK of dlPA. A change in the ionization of the substrate (which would depend on the surface pressure) may influence the enzymatic activity. However, positive deviations of the surface potential per molecule are also found for mixed films of gangliosides with dlPA and dlPC [15]. In these cases, the enzyme is inhibited at all pressures. Thus, our results can not be explained only by a correlation with the ionization state of the interface. The presence of Sulf abolishes the two-dimensional phase transition of dlPA at 20–22 mN · m^{-1} [15]. It also eliminates the abrupt increase of enzyme velocity found in films of pure dlPA above 18 mN · m^{-1} (Fig. 3). The interactions among Sulf and dlPA are unfavorable. This is revealed by positive excess free energies of mixing and lateral immiscibility at certain proportions and pressures [15]. This may explain increased enzyme velocities due to inhomogeneous regions in the mixed film.

Ganglioside G_{M1} (at molar fraction 0.25) inhibits PLA_2 activity against dlPA with a maximum at 20 mN · m^{-1} (Fig. 3). The more complex gangliosides G_{D1a} and G_{T1b} abolish the enzyme activity at all pressures at molar fractions of 0.25 and 0.15, respectively. The effects of gangliosides on the enzyme activity toward dlPA are qualitatively similar to their influence on the hydrolysis of dlPC by PLC (Fig. 2) or by PLA_2 [14] (Table II). Different to films with Sulf, the interactions of both dlPA and dlPC with gangliosides are thermodynamically favorable [15]. Gangliosides induce marked reductions of the mean molecular areas at all pressures in these films. The effects are more marked at higher surface pressures and molar fractions of gangliosides. The interface becomes more liquid-condensed and less compressible than that of pure dlPA [15]. In addition, the two-dimensional phase transition of this lipid at 20–22 mN · m^{-1} does not occur in the presence of gangliosides. At this pressure the activity of the enzyme against monolayers containing gangliosides is null. The favorable tight packing of the phospholipid substrate with gangliosides, especially at high surface pressures may partially account for the interference with enzyme activity.

Gangliosides could desorb from the monolayer and form micelles that might conceivably trap the enzyme in the subphase, leading to an apparent inhibition. However, the constancy of the surface potential on the reaction compartment is against this possibility. Also, no loss of monolayer components occurs in films of pure gangliosides or mixed with phospholipids (see Materials and Methods and Refs. 15, 17 and 28). The presence of proteins in the system does not modify this behavior [29]. Moreover, the loss of a monolayer component should lead to a decrease of the surface pressure. This would be immediately compensated by the surface barostat and reflected as an apparent activation rather than the inhibition observed. As previously suggested [14], the inhibitory effect of gangliosides is not irreversible and is probably mediated by their effect on the surface organization. This is further supported by the following experiments of film transfer.

The technical approach originally described by Rietsch et al [23] was used (Fig 4). The film formed by dlPA/G_{M1} (3:1) was first set at 20 mN · m^{-1} in compartment II; then an appropriate amount of enzyme was injected into the subphase. Due to the presence of ganglioside and the high pressure, no catalytic activity is observed under these conditions (see also Fig. 3). After 10 min, the film is transferred and rinsed in compart-

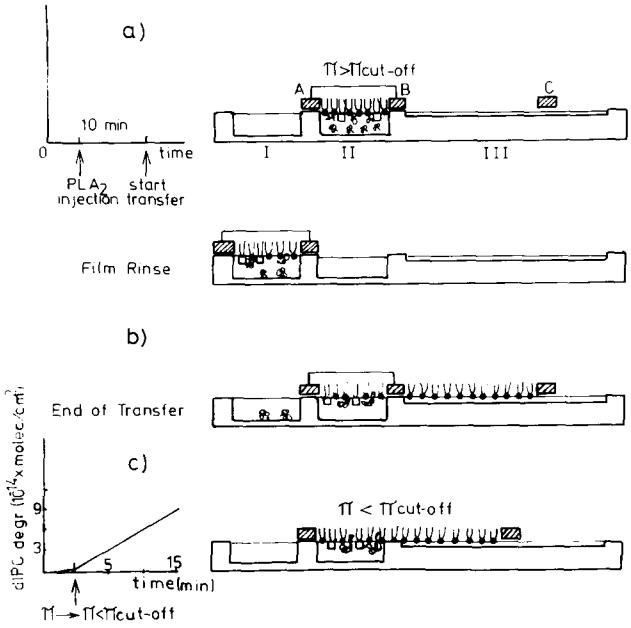

Fig. 4. Adsorption of PLA_2 to phospholipid-ganglioside mixed monolayers Effect of film transfer. (a) At time zero, 0.025 μg/ml (final concentration) of PLA_2 is injected beneath a mixed film of $dlPA/G_{M1}$ (3:1) set at 20 $mN \cdot m^{-1}$ and limited by barriers A and B. (b) After 10 min of continuous stirring the mixed film is transferred and rinsed in compartment I, and again transferred to the reaction compartment II. (c) The barrier B was removed, the surface pressure was rapidly adjusted to 15 $mN \cdot m^{-1}$, and PLA_2 activity was recorded.

ment I and again transferred to compartment II at constant pressure (Fig. 4). Then the surface pressure is rapidly (within 30 s) reduced to 15 $mN \cdot m^{-1}$ (if the enzyme remained associated to the monolayer during transfer it should be able to hydrolyze the substrate at this pressure, see Fig.3). After this procedure we observed enzymatic activity with a velocity of about $0.6 \cdot 10^{14}$ molecules $\cdot min^{-1} \cdot cm^{-2}$. A similar behavior (not shown) was obtained with a film of $dlPC/G_{M1}$ (2:1) initially set at 12 $mN \cdot m^{-1}$ (no enzyme activity), transferred at the same pressure, rinsed and rapidly set to 7 $mN \cdot m^{-1}$ (inhibited but clearly observable enzyme activity, see also Ref. 14). The reaction rates measured in these experiments were about 40% smaller than those obtained at the same surface pressure but without film transfer. This may be due to diminished amounts of enzyme interacting with the film at the relatively high initial surface pressure and, most probably, to unavoidable film losses during transfer. However, these experiments clearly demonstrate that the enzyme did adsorb to the interface in the presence of gangliosides at high pressures but it was not able to show catalytic activity. This is probably due to an unsuitable interfacial organization. After film transfer and reducing the pressure to 15 $mN \cdot m^{-1}$ the activity of the enzyme starts immediately, without a measurable latency period.

Additional experiments were performed under conditions of variable ganglioside composition at the interface. This was achieved by spreading the same mixture of GSLs-dlPC on the interface of both the reaction compartment and the reservoir. In this condition there is a continuous enzymatic depletion of dlPA while the molar fraction of the GSLs progressively increases in the film. PLA_2 exhibits the reported diminished velocity [14] with a film of $dlPC/G_{M1}$ (2:1) at 10 $mN \cdot m^{-1}$ compared to pure dlPC (Fig. 3, inset). Nevertheless, the enzyme could degrade 100% of the substrate offered, albeit at the reduced rate. We wish to emphasize that the enzyme exhibits no activity at 10 $mN \cdot m^{-1}$ against a film of $dlPC/G_{M1}$ with an initial molar fraction of G_{M1} of 0.5 (see Table I and Ref. 14). If irreversible inhibition of the enzyme due to binding to the ganglioside had occurred, the degradation of dlPC should have halted when the molar fraction of ganglioside in the film reaches the value of 0.5. This is not observed, the enzyme can continue the degradation of dlPC up to 100% if it was injected beneath a film of $dlPC/G_{M1}$ with an initial ganglioside molar fraction of 0.33. This demonstrates that even if the enzyme might have been bound to gangliosides it could clearly act on the substrate as soon as the interfacial organization becomes suitable. This can not be due to a more favorable substrate competition for the enzyme since its proportions in the film is continuously decreased under the kinetic regime employed. These effects are currently under more detailed study.

General considerations

The changes of surface potential depend linearly on the amount of enzyme in the system [14] and are similar for systems showing different rates of activity (Table I and II). Taken together, our results suggest that the initial interaction of the enzyme with the interface is not markedly affected by gangliosides. These lipids may, however, interfere with the accessibility of the enzyme to the phospholipid acyl-ester region or the attainment of a suitable organization of the substrate (or the enzyme). The ganglioside oligosaccharide chain protrudes into the aqueous phase perpendicular to the surface; the sialosyl residues are located at least 1 nm away from the interface [10,28,30,31]. If the enzyme interacts with this region, it may remain in an unsuitable position to reach the phospholipid ester bond to be cleaved. By contrast, adsorption to interfaces containing Sulf with a negatively charged but shorter polar head group leads to enzyme activation [14]. The effect of gangliosides on the activity are not irreversible and the intermolecular organization of the substrate is markedly affected [15,17]. The presence of monomeric gangliosides in the subphase (below their CMC but still in molar ratios over 10:1 with respect to the enzyme) do not modify the phospholipase activity toward a pure dlPC film [14]. The experiments in which the surface composition is

continuously varied at constant pressure are also against an irreversible inactivation due to enzyme binding to gangliosides.

The local magnitude and orientation of the polar head group dipole moments as well as a modified interfacial hydration due to GSLs may be involved in the modulation of the phospholipase activity. The electric field across the monolayer interface affects reversibly the activity of PLA_2. The enzyme is activated with negative potentials above the monolayer while its activity is decreased with positive potentials [32]. The activating field hyperpolarizes the dipole moment vector of phospholipids (positive toward the hydrocarbon chain methyl groups Ref. 33) while the inhibitor field depolarizes it.

The oligosaccharide chain of gangliosides and complex neutral GSLs contributes with a considerable resultant dipolar component. The positive terminus of this dipole points toward the subphase approximately perpendicular to the interface and opposite to the hydrocarbon chain dipole moment vector [10,11,28,31]. This decreases the total ganglioside dipole moment and makes the surface potential less positive [15,17]. This effect is operationally similar to a local depolarization of the interface. Qualitatively, the variations of phospholipase activity to be expected according to the dipolar properties of gangliosides are in keeping with the results observed by directly applying electrical fields [32].

On the other hand, it is known that a dehydration process occurs at the interface when PLA_2 interacts with phospholipid bilayers [34,35]. The microenvironment of ganglioside-containing interfaces is more polar and hydrated [36–38]. The inhibitory action on the phospholipase activity by gangliosides (and complex neutral GSLs such as asialo-G_{M1}, see Ref. 14) may be enhanced by their highly hydrated polar head groups. The combination of an unsuitable local electrical field, an increased thermodynamic resistance of the interface to be dehydrated [39] and a tighter packing with the substrate molecules [15,17] are all factors that may modulate the degradation of phospholipids by phospholipases in interfaces containing GSLs. These factors can be dynamically modified by the conditions in the membrane itself and in the aqueous environment [10]. Our findings can not be extrapolated directly to the case of membrane bound or intracellular phospholipases able to trigger a cascade of cellular responses mediated by lipid-derived second messengers [40]. Practically nothing is known on how the membrane organization affects their activity. However, similar to all the phospholipases studied so far, surface effects of the type described also affect the activity of a brain phosphatidylinositol-specific PLC [41]. Conceivably, gangliosides could be involved in the modulation of these phospholipases in view of their marked effects on membrane protein phosphorylation [13], protein kinase C activity [42], cell development [43] and transformation [44].

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