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Regulation by gangliosides and sulfatides of phospholipase A_2 activity against dipalmitoyl- and dilauroylphosphatidylcholine in small unilamellar bilayer vesicles and mixed monolayers

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Abstract

The modulation by gangliosides GM1 and GD1a, and sulfatide (Sulf) of the activity of porcine pancreatic phospholipase A_2 was studied with small unilamellar vesicles of dipalmitoylphosphatidylcholine (L-dpPC) and lipid monolayers of dilauroylphosphatidylcholine (L-dlPC). The presence of Sulf always led to an increase of the maximum rate of the enzymatic reaction, irrespective on whether the vesicles were above, in the range of, or below the bilayer transition temperature. Sulf did not modify the latency period for the reaction that is observed at the bilayer phase transition temperature. Gangliosides inhibited the maximum rate of enzymatic activity against bilayer vesicles in the gel phase but the effect was complex. When the reaction was carried out at a temperature within the range of the bilayer phase transition, the gangliosides inhibited the maximal rate of the reaction in proportion to their content in the bilayer. However, at the same time the latency period observed with vesicles of pure phospholipid at this temperature was shortened in proportion to the mole fraction of gangliosides in the bilayer. At temperatures above the bilayer phase transition, gangliosides stimulated the activity of PLA_2. Preincubation of the enzyme with Sulf or gangliosides did not affect the activity against bilayer vesicles of pure substrate. These glycosphingolipids did not modify the rate or extent of desorption of the enzyme from the interface, nor the pre-catalytic steps for the interfacial activation of PLA_2, or the enzyme affinity for the phospholipid substrate. Also, the activity of the enzyme was not altered irreversibly by glycosphingolipids. Our results indicate that Sulf and gangliosides modulate the catalytic activity of PLA_2 at the interface itself, beyond the initial steps of enzyme adsorption and activation, probably through modifications of the intermolecular organization and surface electrostatics of the phospholipid substrate.

Key words: Glycosphingolipid; Ganglioside; Sulfatide; Phospholipase A_2; Monolayer; Unilamellar bilayer vesicle

1. Introduction

Details of various molecular events responsible for the complex interfacial modulation of the activity of phospholipases from various sources have been unravelled by different groups of investigators. Most of the mechanisms proposed to explain the complex surface kinetics observed are consistent with the existence of two major and independent steps for the enzymatic reaction. These are related to the initial adsorption of the enzyme to the interface and to its subsequent activation leading to phospholipid hydrolysis. These two steps are markedly regulated at the molecular level by the initial phase state of the lipid, by the formation of lateral defects or immiscible surface domains, by the presence of the products of hydrolysis, and by the intermolecular organization or local conformation of the phospholipid substrate [1–10].

We have previously described the effect of several

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Abbreviations: GSLs, glycosphingolipids; Cer, ceramide (*N*-acylsphingoid); NeuAc, *N*-acetylneuraminate; Sulf, Gal(3-sulfate)β1–1Cer; G_{M1}, Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1Cer; G_{D1a}, NeuAcα2–3Gal β1–3GalNAcβ1–4Gal(3–2αNeuAc)β1–4Glcβ1–1Cer; L-dpPC, L-dipalmitoyl phosphatidylcholine; D-dpPC, D-dipalmitoylphosphatidylcholine; L-dlPC, L-dilauroyl phosphatidylcholine; SUV, small unilamellar vesicles; PLA_2, porcine pancreatic phospholipase A_2.

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glycosphingolipids (GSLs) on the activity of porcine pancreatic phospholipase A_2 (PLA_2) and *Clostridium perfringens* phospholipase C (PLC) against monolayers of dilauroylphosphatidylcholine (L-dlPC) and dilauroylphosphatidic acid (L-dlPA). The rate and extent to which these phospholipases can degrade the phospholipid is markedly dependent on the type and relative proportion of GSLs present at the interface, and on the lateral surface pressure [11–13]. Recently we have further found that the PLA_2 activity is modulated in a concerted manner by the relative proportions and interactions of sulfatide (Sulf) and myelin basic protein in the monolayer [14].

We suggested previously [11–14] that there are at least two levels at which the modulatory effect of GSLs may be exerted: one is on the initial interaction of the enzyme with the interface; another controls the expression of activity at the surface itself probably through the molecular organization of the substrate, or of the steps related to the enzyme activation subsequent to adsorption. In the present work we have focused on the changes induced by Sulf and gangliosides GM1 and GD1a on the initial steps of the reaction catalyzed by PLA_2 with small unilamellar vesicles of dipalmitoylphosphatidylcholine (dpPC) and lipid monolayers of dlPC. Our results support the notion that the action of these GSLs is exerted at the level of the intermolecular organization of the substrate.

2. Experimental procedures

Purification of Sulf and gangliosides GM1 and GD1a from bovine brain was described previously [15–17]. L-dpPC and L-dlPC were from Avanti Polar Lipids (Birmingham, AL), D-dpPC was from Sigma (St. Louis, MO). Porcine pancreatic PLA_2 was from Boehringer-Mannheim (IN, USA), diluted 1:50 with 50 mM KCl, dialyzed 4 h against 400 vol. of this solution, and adjusted to pH 8.0 with KOH. Over 95% of the protein ran as a single band of 14 kDa on SDS-PAGE. The enzyme concentration was determined at 280 nm, $\epsilon_M = 13.0$ [18]. No surface active compounds were detected in the enzyme solution at concentration 10 times higher than that used in the assays. Deionized, all-glass bidistilled water was used. Absence of surface active impurities in solvents and salts was routinely checked as previously described [17].

2.1. Bilayer vesicles

Small unilamellar vesicles (SUV) of phospholipids containing GSLs were prepared as described before [19–21]. Briefly, the solvent was evaporated under N_2 and the dry material was kept at 45°C under vacuum for at least 4 h. The lipids (40 mM) were hydrated for 1 h at 50°C with 50 mM KCl, containing 1 mM sodium azide, mechanically dispersed for 3 min, sonicated (35% power output) at 50°C, under N_2, for 20 min (with 1 min pulse delay) and centrifuged at 15 000 × *g* for 30 min to remove undispersed lipid. The vesicles were dialyzed for a minimum of 4 h against 400 vol of an equi-osmolar solution of 35 mM KCl and 10 mM $CaCl_2$, adjusted to pH 8.0, for equilibration of Ca^{2+} across the vesicle membrane. The effects of GSLs on the PLA_2 activity were unchanged for vesicles prepared by sonication in the presence of 10 mM $CaCl_2$. However, in this case we found less reproducibility of enzymatic activity (as well as of light scattering and lipid hydration, see Refs. [16,38]) and the first procedure was the one routinely used. The vesicle dispersion was always kept at 45°C to avoid vesicle fusion. Entrapment of carboxyfluorescein (50 mM) solution [20,21] was between 7.2 and 10.7 nmol/μmol of lipid which corresponds to an entrapped volume of the original solution of 0.14 to 0.21 μl/μmol lipid. This is in keeping with the expected volume of vesicles having the diameters found (negative staining EM with 1% uranyl acetate): average vesicle diameter for pure L-dpPC was 35 ± 10 nm, and for 10 mole% of Sulf, GM1 and GD1a were 40 ± 15 nm, 30 ± 10 n 20 ± 5 nm, respectively [19,21]. Less than 10% variation of light scattering at 450 nm occurred over a period of two days, control experiments with vesicles loaded with carboxyfluorescein showed maximum leakage of the order of 4%. The vesicle light scattering, carboxyfluorescein fluorescence, and the changes of intrinsic (tryptophan) fluorescence of PLA_2 were measured in a SLM-4800C spectrofluorometer (SLM Instruments, Urbana, IL) as described elsewhere [20,21]. No detectable spontaneous fusion occurred among the vesicles when used within two days.

The time-course of hydrolysis of L-dpPC by PLA_2 was determined with a Mettler DL21 autotitrator (1-ml burette) in the pH-stat mode with carbonate-free 0.01 M NaOH, in KCl 0.05 M titrated on the same day of the experiments against 10 mM potassium biphthalate, under N_2. All titrations were carried out in 1.5 ml with continuous stirring under N_2. Temperature was controlled (±0.2°C) with a thermostatized cell block (SPR-5 controller) and KPC-5 temperature programmer (Shimadzu, Japan). The titrant and reaction solutions were degassed and pre-titrated to pH 8.0, after 10 min, 25 μl of the dialyzed enzyme solution at pH 8.0 was injected into the cell and the consumption of NaOH determined as a function of time. The variation of the pH during the reaction was within ±0.05 pH units. The rate of titrant consumption before enzyme addition represented less than 0.05 μmol of NaOH in 10 min and was always subtracted. The results shown in this work are the average of duplicate or triplicate experiments. Reproducibility of enzyme velocities and

lag-times for experiments repeated with the same vesicle preparation was within ±10%. However, variations among experiments done with different batches of vesicles prepared in different days could be as much as ±20%. This has been noted before [22] and is most likely due to the extreme sensitivity of PLA_2 to detect slight changes of substrate organization induced by small variations of conditions during the preparation of SUV. For this reason, control reactions with vesicles of pure L-dpPC were always included in each set of experiments. The relative differences of behavior among vesicles containing GSL and pure phospholipid was consistent for experiments run with vesicles prepared at the same time, kept at 45°C and used within two days.

2.2. Lipid monolayers

Monolayers of pure L-dlPC or mixed films with GSLs were prepared and monitored as previously described [13,14]. The equipment was a Monofilmmeter (Mayer Feintechnik, Gottingen, Germany) in which the original circular trough was replaced by one specially designed. The teflon-coated trough has several compartments of different surface area and volume, connected through narrow and shallow slits (5 mm wide × 5 mm long × 1 mm deep). The surface potential was measured with a Corning ionalyzer 250 millivoltmeter and an ^{241}Am air-ionizing (5 mm above the surface) and calomel (subphase) electrodes. The signal from the millivoltmeter, the one corresponding to the surface area and the surface pressure (platinized Pt foil 12.5 mm wide × 20 mm long × 0.025 mm thick) were fed into a double channel *X-Y-Y* recorder (Yokogawa Corp., Japan). The surface pressure can be automatically maintained constant on any compartment by a compensation circuit servo-operating the compression barriers. Before each experiment, the trough was wiped clean and rinsed with 70% ethanol and twice with bidistilled water. After spreading 10–20 μl of the lipid solution in chloroform/methanol, 5 min were allowed for solvent evaporation and the films were compressed to the desired initial surface pressure which was then maintained constant. The desired amount of enzyme (in no more than 50 μl) was injected into the subphase solution (17 to 34 ml) of 10 mM Tris-HCl buffer, 20 mM $CaCl_2$, 100 mM NaCl (pH 8.0), under continuous magnetic stirring, at 30 ± 0.2°C. When needed, the monolayer was transferred, at constant surface pressure, onto different compartments containing enzyme-free solutions. Zero-order kinetics were achieved as reported before [11,12] by spreading a monolayer of pure L-dlPC, at the same surface pressure onto an adjacent reservoir. Constancy of film composition was continuously monitored by the surface potential which permits detection of less than 3% variation in the amount and quality of molecular dipoles [3,23–25]. Changes of surface potential were also used to monitor interfacial adsorption of PLA_2 [11]. The values of enzyme velocity are corrected and normalized as previously described [11,13] to account for substrate dilution by the GSLs and for any deviation in molecular packing caused by lipid interactions. Reproducibility for the molecular area, surface pressure and surface potential was ±0.03 nm^2, ±0.5 mN/m and ±10 mV. Recovery of monolayers at selected times, after enzyme adsorption and film transfer, was done with hydrophobic paper (Whatman PS-1) [26]. In control experiments over 95% of the monolayer of L-dlPC labelled with 1% [^{14}C]oleic acid was recovered. A maximum of 6 ± 2 $\mu l/cm^2$ of subphase solution (contaning [^{14}]C-sucrose) was collected on the paper. Protein was determined with *o*-phthalaldehyde [26,27] after elution in a small Petri dish with 10 mM Tris-HCl buffer in 100 mM NaCl (pH 8.0) containing 0.15% Brij 35. In a few experiments enzyme activity was assayed after adsorption. Five to ten monolayers were collected, pooled and eluted as described above. The amount of protein recovered was between 1–4 μg of protein. This was concentrated in Centriprep tubes (Amicon) and dialyzed against buffer without Brij 35. The enzyme activity was determined against a monolayer of L-dlPC at 12 mN/m [11–13].

3. Results

3.1. Activity against bilayer vesicles

Fig. 1 shows that the effects of Sulf and gangliosides on the activity of PLA_2 against small unilamellar vesicles of L-dpPC have the same trend as those previously found with lipid monolayers of L-dlPC [11–13]. The presence of Sulf induces an increased rate of PLA_2 activity while the enzyme velocity is decreased with vesicles containing gangliosides GM1 or GD1a. In agreement with previous reports [22,28,29] no lag-time is observed for the reaction with this phospholipid at 22°C.

When an excess of enzyme with respect to the vesicles was used, the total proportion of phospholipid degradation is about 60–65% for pure dpPC. On the basis of the overall asymmetry of vesicles of this size [19,21,30,31], the amount of hydrolysis approximately corresponds to the degradation of all the phospholipid present on the outer vesicle surface. The enzyme velocity increased when the amount of dpPC was higher but, in conditions where the enzyme was still in excess with respect to the vesicles, the proportion of the total phospholipid hydrolyzed was the same. The presence of GSLs increased the extent of dpPC degraded to between 70–80% of the total phospholipid. This is in

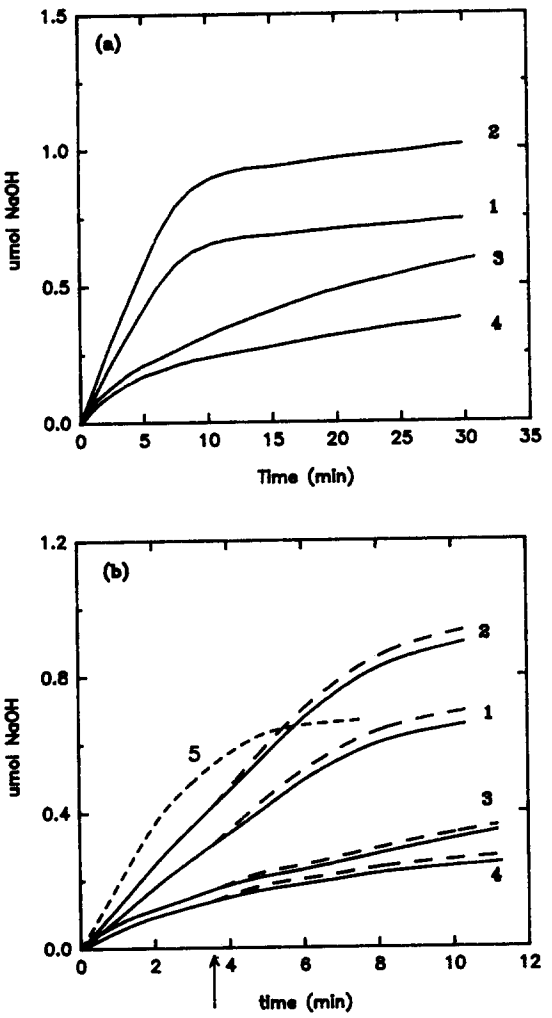

Fig. 1. Activity of PLA_2 against bilayer vesicles of L-dpPC. The amount of phospholipid (1.2 μmol in 1.5 ml) degraded by 6 μg of PLA_2 at 22°C as a function of time is shown in (a) for SUV of pure L-dpPC (1), and for vesicles containing 10 mole % of Sulf (2), GM1 (3) and GD1a (4). Double reciprocal plots of the data (not shown) indicate a total hydrolysis of 62% in SUV of pure L-dpPC, 76% in SUV with GM1, 72% in SUV with GD1a, and 79% in SUV with Sulf. Part b shows the variation of enzymatic activity found (dashed lines) by doubling (at the time indicated by the arrow) the amount of vesicles of pure L-dpPC (1) or containing Sulf (2), GM1 (3) or GD1a (4) during the time of active hydrolysis. Curve 5 shows the activity against 2.4 μmol of L-dpPC before addition of the enzyme; in similar conditions, vesicles containing Sulf or gangliosides also showed approximately doubling of the respective rate of activity shown in part a.

keeping with the known increased capacity of this enzyme to degrade phospholipids in negatively charged interfaces [1,39]. However, the total extent of degradation was independent on the effect of GSLs on the rate of PLA_2 activity (i.e., enhancement for Sulf, or inhibition for gangliosides, see Fig. 1a). It was shown previously in lipid monolayers that the rate and the extent of PLA_2 activity are modulated differently and independently by the GSLs [13].

In experiments where the dpPC/PLA_2 ratio was adjusted so that there was an excess of vesicles with respect to the enzyme a 2-fold increase in the amount of phospholipid vesicles, added either during the linear hydrolytic phase (Fig. 1b) or after the reaction had reached the plateau level (not shown), produced less than 12% of increase in the rate of degradation in all the preparations. By contrast, when the concentration was double from the beginning, before adding the enzyme, the rate of hydrolysis was increased about 2-fold. These results indicate that, in these conditions, the enzymatic reaction is being carried out on the surface of the vesicle and that the enzyme does not exchange, within experimental error, from a vesicle to another during the time of the experiments. This type of behavior (denoted the 'scooting mode' as opposed to the 'hopping mode') has been described before for various phospholipids and phospholipid analogues [1,8].

In the experiments shown in Fig. 2a, the total bulk concentration of L-dpPC and GSLs is increased with respect to the enzyme while the ratio of GSLs and L-dpPC is maintained constant in the vesicle. Each point represents the average of two separate experiments. The initial velocity of the enzymatic reaction at 22°C exhibits classic hyperbolic behavior, in agreement with other authors [1,22,28,29]. However, with these experiments only values for an operational or 'apparent' binding constant (denominated K_{app}) of PLA_2 for the lipid interface may be obtained (cf. Ref. [28]). This is a complex quantity since it is measured through the formation of products and it therefore includes all the initial steps (adsorption, activation) of the catalytic reaction. It is not possible to derive meaningful values for K_M from the curves in Fig. 2 because in the conditions used there is practically no desorption of the enzyme from the surface during the experiment. Thus, the enzyme velocity is only dependent on the relative amounts of enzyme and substrate (diluted by the non-substrate GSLs) at the vesicle interface but it is independent on the excess substrate in the solution [1,8,29]. Nevertheless, the results in Fig. 2 (see Table 1) suggest that the apparent initial interaction of the enzyme to the interfaces containing GSLs may actually be favored (lower K_{app}) independent on their effect on the reaction rate (higher for vesicles with Sulf and lower with gangliosides, compared to L-dpPC).

During the reaction, the proportion of GSLs increases continuously in relation to the substrate at the vesicle surface but the hydrolysis proceeds at an increased (for vesicles with Sulf) or decreased (for vesicles with gangliosides) rate. However, Fig. 2b shows that the percentage of activation (for Sulf) or inhibition (for gangliosides) of the enzyme velocity, at each substrate concentration, is independent on the total amount of lipid present. As shown by Jain et al. [32], this means that the effects of GSLs on the activity of

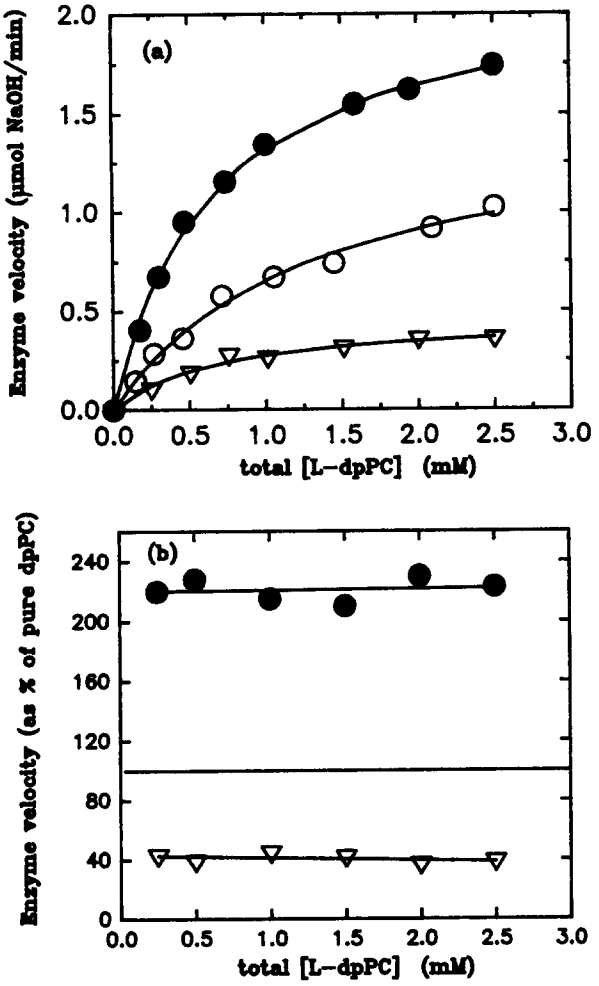

Fig. 2. Variation of the rate of PLA_2 activity with the total amount of substrate. The initial rate of enzymatic activity (6 μg of PLA_2 in 1.5 ml) at 22°C as a function of the total amount of L-dpPC is shown in (a) for SUV of pure L-dpPC (○) and for SUV containing 10 mole% of Sulf (●) and GD1a (▽). The symbols represent the experimental points and the lines are fittings to a Michaelis-Menten equation. The parameters of the fitted curves are shown in Table 1. The percentages of activation or inhibition of enzyme velocity in SUV containing GSLs are shown in b as a function of the total substrate concentration. The value for pure L-dpPC at each point is taken as 100% (horizontal line at 100%).

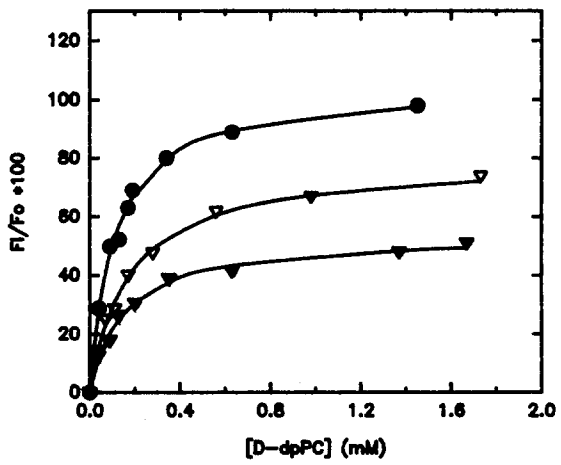

Fig. 3. Effect of GSLs on the fluorescence of PLA_2 upon binding to SUV. The percentage increase of fluorescence intensity of PLA_2 (1 μM) at 22°C is shown as a function of the total amount of D-dpPC (▽) and for SUV containing 10 mole% of Sulf (▼) and GD1a (●). The excitation was at 280 nm and emission was measured at 340 nm. The parameters obtained from the regression lines are given in Table 1.

PLA_2 are occurring at the interface itself and are not due to a change of the absorption equilibrium of the enzyme to the interface. These results are in agreement with previous experiments in monolayers suggesting that GSLs did not affect the enzyme molecules in the bulk solution and that these lipids did not impair interfacial adsorption of the enzyme [12,13].

Fig. 3 shows the change of intrinsic fluorescence of PLA_2 upon binding to vesicles of the non-hydrolyzable substrate analogue D-dpPC. This was used instead of the normal substrate L-dpPC in order to eliminate the perturbing effect of turbidity caused by formation of Ca^{2+} salt of the fatty acid formed during the reaction. The changes of intrinsic fluorescence of PLA_2 upon binding to the normal substrate L-dpPC were qualitatively similar when measured in a medium without Ca^{2+} and 5 mM EDTA. However, we found more irreproducibility in these experiments that in those

Table 1
Kinetic parameters for the interaction of PLA_2 with SUV of dpPC

Vesicle	From Fig. 2		From Fig. 3		From Fig. 4	
	K_{app} (mM)	V_{Mapp} (μmol/min)	K_{mf} (mM)	F_{max} (rel. units)	K_M (mM)	V_{max} (μmol/min)
L-dpPC	1.35	1.51	0.17 * (0.30)	81 * (74)	1.83	1.75
L-dpPC, Sulf	0.65	2.20	0.16 * (0.24)	54 * (44)	1.90	2.15
L-dpPC, GM1	0.69	0.72	0.13 * (0.22)	92 * (83)	1.80	1.23
L-dpPC, GD1a	0.71	0.47	0.10 * (0.17)	105 * (89)	1.85	1.05

* Values for D-dpPC; the values for L-dpPC are shown in parenthesis (see text). The proportion of GSLs in the vesicles is 10 mole% for the experiments shown in Fig. 2 and 3. Different amounts of GSLs were used to achieve surface dilution of L-dpPC for the experiments shown in Fig. 4 (see text). Reproducibility of the values of the kinetic parameters was (in maximum % of the average value): ±15% for K_{app} and V_{Mapp}; ±11% for K_{mf} and F_{max}; ±10% for K_M and V_{max}.

with D-dpPC, which we attribute to variations of light scattering due to residual hydrolytic reaction. The values of lipid concentration required to reach half-maximum fluorescence changes are between 0.11 mM and 0.17 mM for all the vesicles containing D-dpPC and GSLs (Table 1). The maximum relative fluorescence intensities of PLA_2 interacting with vesicles containing Sulf or GD1a are respectively lower and higher than the % F_{max} found with pure D-dpPC (Table 1). The results using L-dpPC in the presence of EDTA were in the similar direction (not shown). These results are in keeping with our previous suggestions [11–14] on the possible lack of interference by GSLs on the initial interaction of PLA_2 with the phospholipid interface, irrespective of the subsequent effect of these lipids on the enzyme activity [11–14] (see also Fig. 2). The changes of fluorescence intensity of PLA_2 and of K_{mf} upon binding to vesicles containing GSLs are inversely correlated to the effect of these lipids on the enzyme activity. The highest fluorescence intensities are found with vesicles containing ganglioside GD1a that inhibits the rate of PLA_2 activity while the lowest intensities are found in vesicles with Sulf that increases the enzyme velocity. This is contrary to effects reported previously for different phospholipids in that the substrate more susceptible to hydrolysis was the one causing the highest fluorescence emission of PLA_2 [1].

The values for the apparent K_M and V_M for the hydrolysis of L-dpPC in vesicles containing GSLs (Fig. 4) were estimated with vesicles containing Sulf or GD1a in mole fractions ranging from 0.01 to 0.3 to achieve the required concentration of L-dpPC at the outer surface of the vesicles. For determination of each initial velocity value different vesicle preparations were employed in which the mole fraction of GSLs in the vesicles, and the amount of total lipid and enzyme in bulk were adjusted simultaneously to reach the surface dilution of L-dpPC required, while, at the same time, the molar ratio of PLA_2 to phospholipid vesicles was kept between 0.4 and 0.6 to ensure that the enzyme was associated to the vesicle surface (see above). The concentration of L-dpPC present at the outer surface of the vesicle was calculated on the basis of the total mole fraction of GSLs, their asymmetric disposition on the outer and inner bilayer leaflet and the overall average outside/inside asymmetry in these vesicles [19,30].

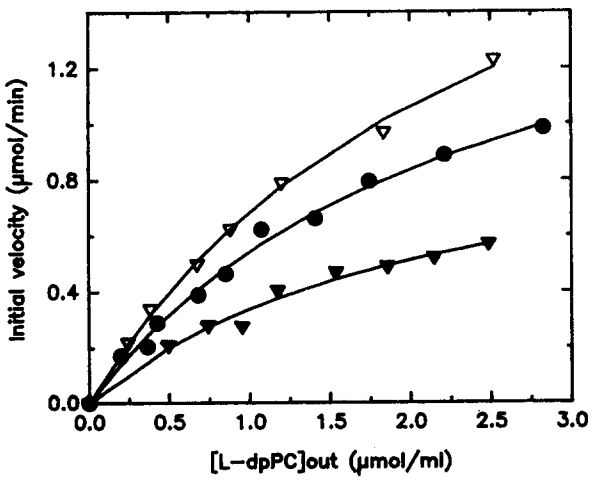

Fig. 4. Variation of the rate of PLA_2 activity as a function of the actual substrate concentration on the outer bilayer surface. The initial rate of enzymatic activity at 22°C is shown for SUV of pure L-dpPC (●), SUV with Sulf (▽) and GD1a (▼). The kinetic parameters obtained from the regression lines are given in Table 1. The molar ratio of PLA_2/L-dpPC was between 0.4 and 0.6 in all experiments, see text.

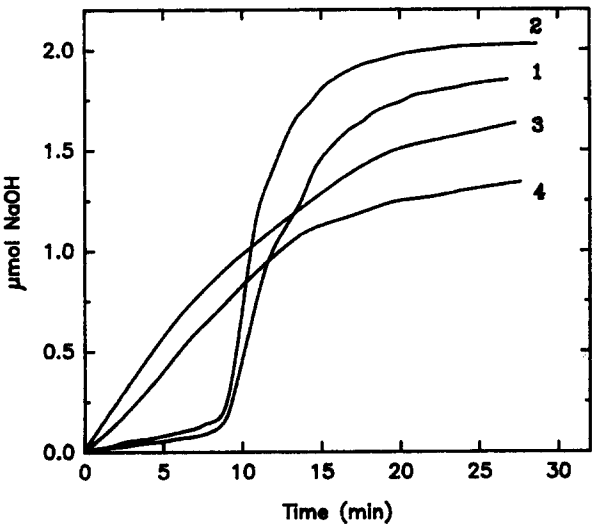

Fig. 5. Activity of PLA_2 against bilayer vesicles of L-dlPC. The amount of L-dlPC (2 μmol in 1.5 ml) degraded by 6 μg of PLA_2 at 22°C is shown as a function of time for SUV of pure L-dlPC (1), and for SUV with 10 mole% of Sulf (2), GM1 (3), and GD1a (4).

The apparent K_M for the hydrolysis of L-dpPC (Table 1) is similar for vesicles of pure L-dpPC and for those containing Sulf that activates PLA_2, or GM1 and GD1a that inhibit PLA_2. The values for V_M were increased by Sulf and decreased by GM1 and GD1a compared to pure L-dpPC. Again, these results indicate that these GSLs do not modify the affinity of the enzyme for the substrate but probably affect a subsequent step that controls the rate of phospholipid hydrolysis.

Fig. 5 shows the effects of GSLs on the activity of PLA_2 against bilayer vesicles of L-dlPC. The presence of Sulf in the vesicle increases the enzyme velocity while gangliosides inhibit it. This is similar to the effects found with vesicles of L-dpPC and those observed previously in mixed monolayers [11–14]. However, different than with L-dpPC, a lag-time for the reaction occurs before the establishment of the maximum rate of activity against vesicles of pure L-dlPC (and those containing Sulf). It is well known that the

occurrence and magnitude of the latency period for achieving maximum enzyme velocity depends on the lipid physical state and on the presence of surface defects that is different for different lipids. The hydrolytic reaction in bilayer vesicles of L-dpPC can occur with no lag-time, with a considerable lag-time, or with no lag-time again depending on whether the temperature is below, in the range of, or above the T_m [1,22,28]. Similarly, note that the reaction with L-dlPC can proceed to almost total phospholipid degradation. Different to vesicles of dpPC, this is probably due to disruption of vesicles formed by short chain phospholipids when the products of the reaction accumulate. The results also show that in vesicles of L-dlPC with gangliosides, even if the maximum rate of hydrolysis is decreased (similar to the effects found with L-dpPC), the lag-time is not present. This unexpected effect induced by gangliosides provided further insights on their mode of action.

To study the striking effect of gangliosides on the lag-time we have subsequently used L-dpPC as substrate because it allows a greater flexibility, in terms of accessible temperatures, for varying the phase state of the lipid. Fig. 6 shows the effect on the latency period of changing the temperature. At 37°C, a latency period of similar duration than for pure L-dpPC is observed with vesicles containing Sulf (Fig. 6a). Similar to the effects at 22°C the presence of Sulf increases the maximum rate of activity but does not affect the lag-time. On the other hand, the presence of gangliosides induces a decrease of the enzyme velocity while abolishing the latency period, similar to the results with L-dlPC described above. As reported by others [22] we found that the activity against vesicles of pure L-dpPC is very low at 48°C. At this temperature, no lag-time is observed for vesicles containing Sulf or gangliosides. Thus, at 48°C, the presence of any of these GSLs leads to an increased enzyme activity against L-dpPC; the enzyme velocity continues to be higher against vesicles containing Sulf than for those with gangliosides.

The presence and duration of the lag-time is related to the initial adsorption of the enzyme to the lipid interface and, mainly, to subsequent activation steps of the enzyme already associated to the interface [1,10,22,29,32]. Our results indicate that GSLs are controlling the surface reaction at the level of the substrate organization. In addition, the findings at 48°C suggest that the pre-catalytic steps may actually be facilitated by any of these negatively charged GSLs. It has long been known that negatively charged interfaces facilitate the activity of this enzyme [1].

In order to investigate more deeply the effect of gangliosides on the lag-time, we studied the activity at 37°C on vesicles containing different proportions of ganglioside. Fig. 7a shows that, at a constant ratio of L-dpPC to PLA_2, the lag-time decreases and is finally

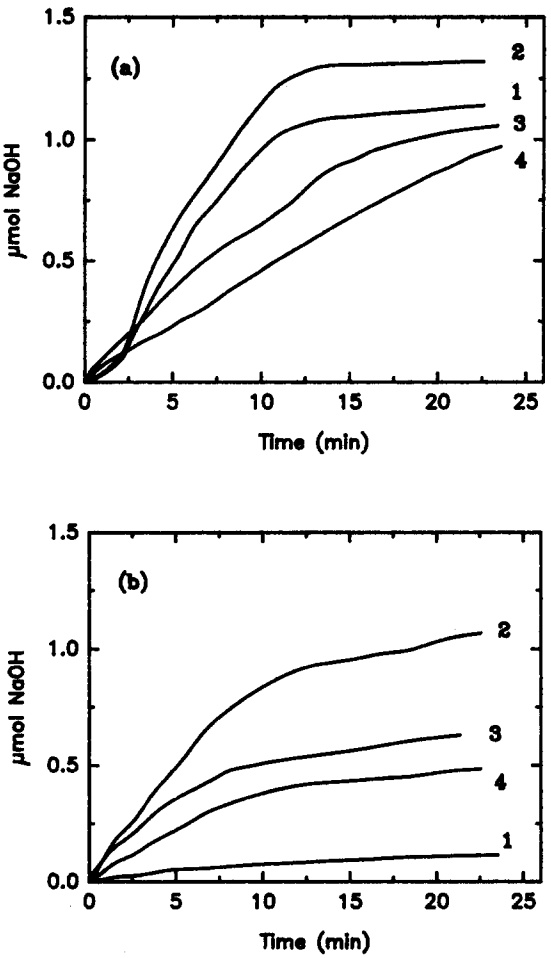

Fig. 6. Activity of PLA_2 against bilayer vesicles of L-dpPC at different temperatures. The amount of L-dpPC (1.76 μmol in 1.5 ml) degraded by 6 μg of PLA_2 at 37°C (a) and at 48°C (b) is shown as a function of time (see also Fig. 1 for comparison with the activity at 22°C) for SUV of pure L-dpPC (1), and for SUV with 10 mole% of Sulf (2), GM1 (3), and GD1a (4).

abolished as a function of the ganglioside mole fraction in the vesicle. The complete elimination of the lag-time requires a greater proportion of GM1 than of GD1a, the effect of both gangliosides being otherwise similar (Fig. 7b). Detailed kinetic experiments have shown that the latency period is directly related to the interfacial activation of porcine pancreatic PLA_2 [1,10,28,33]. It has been reported that several factors that facilitate this step induce a shortening or disappearance of the lag-time [9,28,33]. In agreement with other authors [28], for all the vesicles the latency period increases when the total amount of substrate in relation to the enzyme is increased (Fig. 7c and d). This behavior has been related to a postulated dimerization step that may be involved in the activation of porcine pancreatic PLA_2 prior to catalysis. The similar dependence of the lag-times with the enzyme/phospholipid ratio (Fig. 7d) for all the preparations suggests that gangliosides, though leading to a slower reaction rate, do not impair

the basic nature of the catalytic reaction in the steps assumed to be involved in enzyme adsorption and activation. On the contrary, assuming that the concept of the latency period being a reflection of pre-catalytic steps (cf. Refs. [1,10,28]) is valid for vesicles containing gangliosides, their presence would appear to facilitate enzymatic activation since the lag-time is decreased. The increased enzymatic activity observed in vesicles containing gangliosides at 48°C also suggest that these lipids do not impair the pre-catalytic steps of the reaction.

3.2. Activity against lipid monolayers

The interfacial adsorption and initial activation steps of PLA_2 was further studied using lipid monolayers. In films containing GSLs to which the enzyme is allowed to adsorb at a surface pressure above the cut-off point for activity (after monolayer transfer and rinsing to eliminate enzyme not integrated to the interface, see Refs. [13,14]) the enzymatic reaction starts immediately (without lag-time) when the surface pressure is rapidly brought to values allowing activity [13]. This suggested previously that the GSLs did not affect directly the surface adsorption or activation of the enzyme. On the other hand, the GSLs lead to a different rate or extent of activity of PLA_2 depending on whether the reaction occurs with the enzyme initially accessing the interface from the bulk solution or if it has already been incorporated into the interface [13,14].

Fig. 8a shows the usual pattern of activity against films of pure L-dlPC or containing GSLs under zero-order kinetics: increased activity is seen against monolayers containing 20 mole% Sulf and very little activity is found in the presence of 10 mole% GD1a and GM1 [11–13]. Despite of the modified velocities, Fig. 8 indicates that the amount of enzyme adsorbed to the films (recovered after film transfer and rinsing) is similar in all the monolayers. This shows directly that the GSLs do not modify the initial adsorption step of the en-

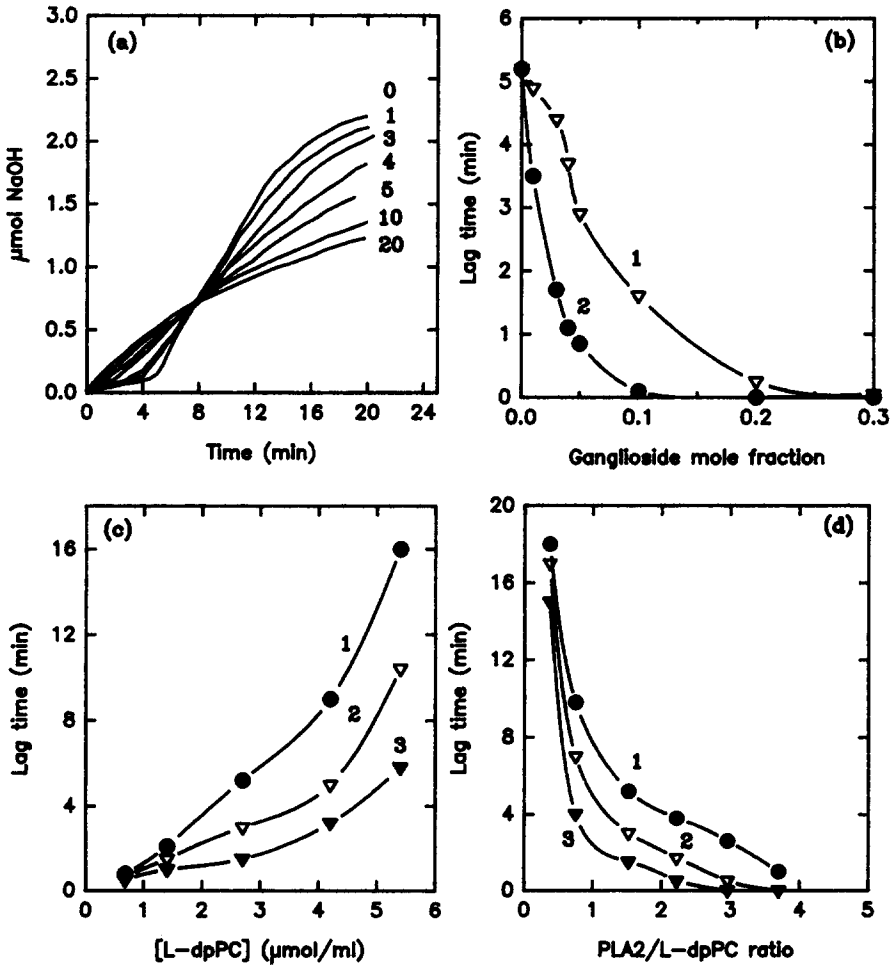

Fig. 7. Effect of gangliosides on the latency period for the degradation of phospholipid vesicles by PLA_2. The amount of L-dpPC (3 μmol in 1.5 ml) degraded by 6 μg of PLA_2 at 37°C is shown in (a) as a function of time for SUV with the mole% of GD1a indicated on the curves. The latency period as function of the mole fraction of ganglioside in the bilayer vesicle is shown in (b) for SUV with GM1 (1) and GD1a (2). The variation of the latency period with the total amount of L-dpPC (c) and the PLA_2/L-dpPC molar ratio (d) are shown for SUV of pure L-dpPC (1) and for SUV with 5 mole% of GM1 (2) and GD1a (3).

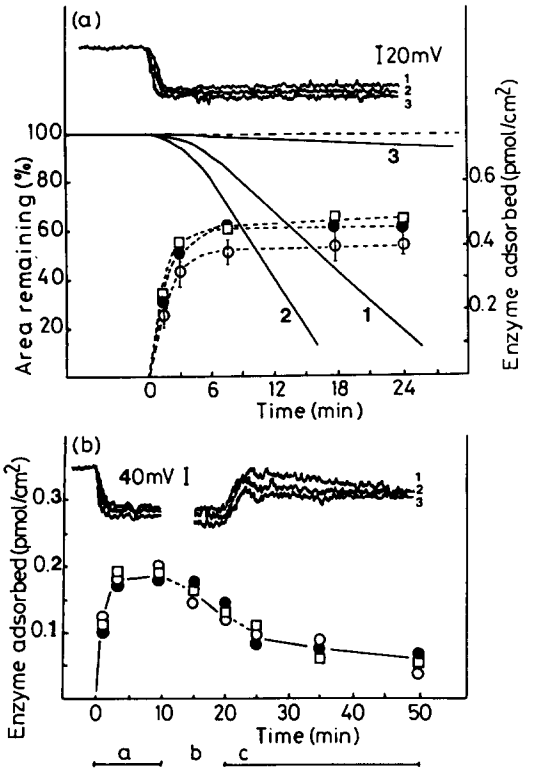

Fig. 8. Activity and adsorption of PLA_2 in lipid monolayers of L-dlPC. (a) Pure L-dlPC was continuously provided at constant surface pressure of 12 mN/m from a reservoir monolayer to achieve zero order kinetics (see Experimental Procedures). The reaction rate (left ordinate) is indicated by the solid lines. PLA_2 (2 nM final concentration in 17 ml subphase) is injected at time zero beneath a monolayer of pure L-dlPC (1), or a mixed film with 20 mole% Sulf (2), or 10 mole% GD1a (3). The wavy tracings in a show the simultaneous changes of surface potential for the monolayers indicated. At selected times, the monolayers were collected and the amount of PLA_2 adsorbed to the film was determined as described in the text. The dashed lines indicate the amount of enzyme recovered (right ordinate) from monolayers of pure L-dlPC (○), and films of L-dlPC with 20 mole% Sulf (●) or 10 mole% GD1a (□). (b) PLA_2 (4 nM final concentration) was injected at time zero beneath a monolayer set above the cut-off pressure for activity (24 mN/m, no degradation occurs for any of the films, cf. Refs. [11–13]) and the enzyme was left to adsorb for 10 min (interval a). The monolayer was transferred and rinsed (at constant surface pressure) over an enzyme-free subphase (interval b). After this, the surface pressure was increased in less than 1 min by 10–12 mN/m above the initial pressure (beginning of interval c). At selected times, the monolayer of pure L-dlPC (○), or the films with 20 mole% Sulf (●), or 10 mole% GD1a (□) were collected and the amount of PLA_2 was determined. The wavy lines in b represent the concomitant changes of the surface potential.

zyme. The changes of surface potential are linearly related to the amount of enzyme adsorbed and reflect the time-course for the interfacial association of the enzyme [11]. For all films, the changes in surface potential show that constant enzyme velocity is reached only after the maximum amount of enzyme was adsorbed to the interface (Fig. 8a).

In principle, the changes of enzymatic activity against monolayers containing GSLs compared to pure L-dlPC may also be due to variations in the rate of interfacial desorption of the enzyme but this is unlikely because the surface potential remains constant and similar in all the films throughout the enzymatic reaction (beyond the initial rapid decrease due to enzyme adsorption [cf. 11]). However, we investigated directly this possibility. The enzyme was first left to adsorb to the film at a pressure of 24 $mN\,m^{-1}$, which is above the critical pressure (cut-off point) over which no enzyme activity is present [11,12]. The adsorption process was monitored continuously by the changes of surface potential and, in independent experiments, by collecting a number of films at selected periods of time and determining the amount of protein adsorbed (Fig. 8b). Subsequently, the film was transferred and rinsed over an enzyme-free subphase after which the monolayer was transferred back, at constant surface pressure, to the initial compartment. The surface pressure was then rapidly (within 30 s) increased by 10–12 $mN\,m^{-1}$ over the initial value in order to induce enzyme desorption [7,34]. The amount of enzyme recovered from the films in parallel experiments was again similar for all the monolayers (Fig. 8b). It can be seen that PLA_2 was lost from the monolayer during film transfer, rinsing and, as reported by others [35], even more after the increase of surface pressure. The surface potential changes reflect the interfacial association and dissociation of the enzyme. The important point to emphasize is that the amount of enzyme initially adsorbed, and then desorbed due to the increase of surface pressure is, within experimental error, similar for all the films. This indicates that Sulf which activates the enzymatic reaction or gangliosides that inhibit it do not modify, within detectable limits, the rate and extent of adsorption and desorption of the enzyme compared to films of pure L-dlPC. Recovery of the enzyme activity in pooled films collected at selected times after adsorption and film transfer (and subsequently measured against monolayers of pure L-dlPC at 12 mN/m) showed that as much as 40% of the activity may be lost, which we attribute to inevitable irreversible enzyme inactivation during film collection and recovery, but again no significant differences were found among films of pure L-dlPC and those containing GSLs.

4. Discussion

Our previous results [11–14] showed that GSLs do not affect directly the enzyme in the bulk solution, nor induce irreversible modification of its activity at the surface, and suggested that these lipids do not impair the interfacial adsorption of the enzyme. Although several details have been elucidated, the precise mechanism for the complex kinetics of surface hydrolysis of

even pure phospholipid substrates by PLA_2 is still not entirely clear [1,8,10,33]. The existence of at least three states of the enzyme in the interface has been proposed [33]: a, an inactive bound state, b, a state with increased fluorescence that possesses little or no activity and c, an active state. Within these possibilities, our results could be compatible with the gangliosides changing the relative proportion of the enzyme between states b and c. However, the effect of lipid activators or inhibitors is difficult to define with simple sequential mechanisms because these enzymes are highly sensitive to synergic effects arising from concomitant changes of the membrane dynamics and organization. These become amplified with the number of components present at the interface. However, it is possible to determine major steps at which the GSLs affect the enzymatic reaction. The present results show that the GSLs modulate the catalytic activity of PLA_2 beyond the initial steps of enzymatic adsorption and activation. The K_M of the enzyme is not significantly modified by Sulf or gangliosides. This indicates that, even if these lipids respectively activate and inhibit PLA_2 activity, the intrinsic enzymatic affinity for the substrate is not affected. On the other hand, the lower values of K_{app} and K_{mf} suggest that both Sulf and gangliosides favor the initial interaction of PLA_2 with the lipid interface, irrespective of their subsequent effect on the rate of activity. The molecular details by which GSLs affect the efficiency of the enzyme may derive from the combination of a variety of factors that are synergistically modified by these lipids in the membrane surface.

Sulf and gangliosides may alter the intermolecular organization (or local conformation) of the phospholipid, the accessibility of the enzyme to the acylester region once it has associated to the interface, or steps controlling the release of products. With respect to the latter, it is relevant that the products of phospholipid hydrolysis (lysolecithin and fatty acids) reduce or eliminate the latency period depending on their proportion in the vesicle [1,10].

Lateral defects due to packing constraints [24,25,36] and formation of phase-separated pure phospholipid domains (with GSLs enriched with respect to their overall mole fraction in localized surface regions) occur at the surface when the proportion of GSLs is relatively low [15,16,19,37,38,50]. There is consensus that surface inhomogeneities facilitate phospholipase binding and the hydrolytic activity begins at these defects [1,5,10,22,28]. It is also known that lateral segregation or enrichment of anionic amphipaths accelerates the enzyme adsorption to bilayers of phosphatidylcholines [1,9,29], and that phospholipase activity is enhanced toward acidic phospholipid substrates, or when negatively charged amphipaths with a small polar head group are present at the interface [1,39].

The oligosaccharide chain of gangliosides protrudes into the aqueous phase with the negatively charged sialosyl residues located at least 1 nm away from the interface [23,40]. Although ganglioside-containing interfaces do not impair the adsorption of the enzyme, if bound to this region, it would be difficult for the enzyme to efficiently cleave the phospholipid ester bond. The reduction or elimination of the latency period before the onset of fast enzymatic reaction against vesicles containing gangliosides may be due to their negative charge and/or surface phase inhomogeneities introduced by the gangliosides that facilitate initial adsorption and activation of PLA_2 (cf. Ref. [5]). On the other hand, the non-ideal variations of molecular packing and dipolar properties induced by gangliosides on the phospholipid [24,25,36] probably interfere with the relative conformational freedom of the substrate required for the enzymatic reaction. Only the reaction velocity is affected but not the total extent of hydrolysis (see above and Ref. [13]) and the results consistently indicate that the ganglioside effect is not due to alteration of the affinity of PLA_2 for the phospholipid or to irreversible substrate or enzyme sequestering but to a reversible impediment that slows down steps regulating the hydrolytic cleavage of the fatty acid moiety, or the formation or liberation of product at the interface. It was shown in monolayers that product formation from long chain phospholipids sequesters PLA_2 in discrete domains where it may become inactive [5]. Conceivably, gangliosides could decrease the desorption rate of these products. However, in monolayers of short chain substrate L-dlPC the products are immediately desorbed from the interface as indicated by the zero-order kinetics and we previously showed that the effect of gangliosides on the rate and extent of the reaction is reversible with the surface pressure [13]; this means that the enzyme does not become irreversibly inactivated in the ganglioside-containing interface.

In Sulf, the negatively charged polar head group formed by only one sulfated carbohydrate is very short [23,41] and this lipid always increases the enzyme velocity. Also, the modifications of phospholipid packing by Sulf are different [25] and these interfaces have considerably more inhomogeneities, especially in the presence of Ca^{2+} [16,38]. This implies that there are enhanced fluctuations of the lateral surface pressure and molecular area [42,43] all of which are effects known to facilitate the enzyme activity [5].

On the other hand, changes of electrostatic polarization due to GSLs-induced alterations in the local magnitude, sign, and orientation of interfacial polar head group dipole moments should not be disregarded in their capacity to modulate enzymatic activity. The electrostatic field across the interface can reversibly affect PLA_2 activity: the enzyme is more active when

the electrostatic field is increasingly negative on the hydrocarbon chain side of the interface while the activity decreases when it is positive [44]. For phospholipids, the positive end of the electrostatic field vector of the resultant molecular dipole moment points toward the end of the hydrocarbon chain. Thus, at the molecular level the imposition of an activating field induces a hyperpolarization of the phospholipid dipole moment while an inhibitory field causes its depolarization. In relation to our results, the oligosaccharide chain of gangliosides contributes with a large local dipole moment opposite to that of the hydrocarbon chains [23,45,46]; this represents a local depolarization of the interface at the molecular level compared to a surface of pure phospholipid [46]. Therefore, the alteration of the local electrostatics induced by gangliosides is in peculiar agreement with the effects of the electrical fields on the enzyme activity.

In addition, it has also been shown that a dehydration step of the interface is required in order to allow an efficient phospholipid hydrolysis by PLA_2 [47]. However, upon binding to small unilamellar vesicles of L-dpPC or L-dpPC with GSLs not only does the emission intensity of Trp-3 of PLA_2 increase but the emission maximum is blue-shifted from 344 to 340 nm. This suggests that the environment of the 'interfacial recognition region' [39] of the enzyme becomes less polar even in the presence of the highly polar and hydrated gangliosides [48,49]. These results and the effects on the lag-times again suggest that the inhibition observed with gangliosides is not due to a sequestering of the enzyme into a more polar environment that would impair its hydrolytic capacity.

The molecular modulation of phospholipase activity by GSLs results from a complex balance of synergic effects taking place in the lipid interface. So far, we have dissected several steps of the surface reaction at which the GSLs do not have a marked influence, and brought into focus that their regulation occurs at the level of the phospholipid organization.

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6. References

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