Modulation of phospholipase A_2 by electrostatic fields and dipole potential of glycosphingolipids in monolayers

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Abstract **Phospholipase A_2 activity against mixed monolayers of dilauroylphosphatidic acid or dilauroylphosphatidylcholine with glycosphingolipids can be reversibly modulated by external constant electrostatic fields. The changes of enzymatic activity are correlated to the depolarization or hyperpolarization of the film caused by specific dipolar properties of glycosphingolipids. Hyperpolarizing fields enhance the enzymatic activity against pure dilauroylphosphatidic acid while depolarizing fields induce a decrease of activity. Compared to the pure substrate, the interface of mixed films containing neutral glycosphingolipids or gangliosides is already partially depolarized and the magnitude of activation induced by an external hyperpolarizing field is decreased; conversely, depolarizing fields cause an increased inhibition of activity. Differing from gangliosides, sulfatides bring about a hyperpolarization of the mixed lipid monolayer and external hyperpolarizing or depolarizing fields cause enhanced activation and reduced inhibition, respectively. The effects of glycosphingolipids depend on their relative proportion in the monolayer. Results were similar with dilauroylphosphosphatidylcholine but the field effects were less than half of those found with dilauroylphosphatidic acid. Our work shows that the activity of phospholipase A_2 in addition to responding reversibly to external electrostatic fields, is directly modulated by the polarity and magnitude of the lipid polar head group dipole moments.—** Maggio, B. **Modulation of phospholipase A_2 by electrostatic fields and dipole potential of glycosphingolipids in monolayers.** ***J. Lipid Res.*** **1999.** 40: **930–939.**

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Evidence has grown steadily showing that constant or alternating electromagnetic fields of different intensity can induce dramatic effects in biosystems (1). This should not be surprising in view of the marked molecular and supramolecular structural anisotropy, which implies electrostatic dipolar anisotropy that is inherent to biological molecules. This occurs from the very basic level of spatially oriented orbitals covalently linked in chemical groups of complex molecules to the long range electrostatic asymmetry of organized supramolecular aggregates and macromolecules constrained by topological restrictions (2, 3).

Anisotropically oriented polarized molecules when exposed to constant or alternating electrostatic fields can respond with different effects. These include dynamic modifications of membrane topology (4–6), cellular function (7, 8), protein phosphorylation (9), transmembrane fluxes of ions and metabolites (10), as well as activation of membrane enzymes that are naturally part of (or become associated to) a lipid interface in order to exhibit activity (11–14). Several of these enzymes participate in cellular energetic metabolism or determine ion fluxes (10, 13–15) while others are part of metabolic cascades that generate lipid second messengers in membrane-mediated signalling (16, 17). This adds at least one important dimension regarding the sensitivity of these enzymes to electrostatic fields of different strength or frequency, namely that transduction or continuation of molecular information can be amplified or dampened according to fields affecting the micro-region of the organized interface in which the enzyme system is thermodynamically or kinetically trapped.

The activity of phosphohydrolytic enzymes such as phospholipase A_2 (PLA_2), phospholipase C (PLC), and sphingomyelinase can be markedly modulated by nonsubstrate sphingolipids such as sphingosine (18), ceramide (19), gangliosides and chemically related glycosphingolipids (19–27). It was previously shown that the control of enzymatic activity by these lipids is exerted at several independent interfacial and topological levels (19, 24–27).

On the other hand, the resultant molecular dipole moment of the oligosaccharide moiety of most neutral glycosphingolipids and gangliosides points toward the opposite direction, to that of the hydrocarbon portion, and its

Abbreviations: PLA_2, phospholipase A_2, porcine phospholipase A_2 (EC 3.1.1.4); dlPA (dilauroylphosphatidic acid) didocecanoyl-*sn*-glycero-3-phosphatidic acid; dlPG (dilauroylphosphatidylglycerol) didodecanoyl-*sn*-glycero-3-phosphatidylglycerol; dlPC (dipalmitoylphosphatidylcholine) didodecanoyl-*sn*-glycero-3-phosphocholine; ceramide, N-acyl-sphingosine; GalCer (galactosylceramide) Glcβ1-1′Cer; asialo-GM1, Gg4Cer, Galβ1-3GalNAc β1-4-Gal β1-4Glcβ1-1′Cer; Sulf (sulfatide 3 O-SO_3-Galβ1-1′Cer; GMl (monosialoganglioside) Galβ1-3GalNAc β1-4-Gal(3-2αNeuAc) β1-4Glcβ1-1′Cer; GDla (disialoganglioside) NeuAcα2-3Galβ1-3GalNAcβ1-4-Gal(3-2αNeuAc) β1-4Glcβ1-1′Cer.

magnitude can be considerable depending on the type and number of carbohydrate residues in the polar head group (3, 28, 29); conversely, sulfatide (Sulf) contributes with an overall polar head group dipole moment that bears a positive end toward the hydrocarbon portion (30). Also, the effects of several glycosphingolipids and some of their chemical or metabolic derivatives on PLA_2 activity can be significantly correlated to their dipolar and packing properties when these are expressed integrated into the magnitude represented by the specific molecular dielectric polarization vector (24). The latter studies were in full agreement with pioneering work showing that the application of direct electrostatic fields of defined strength and polarity can reversibly activate or inhibit PLA_2 activity (12).

PLA_2 is a cationic protein even at pH 8.0 and its activity is very sensitive to negatively charged interfaces containing phospholipids and glycosphingolipids (21); this makes it a potentially useful model for studying the influence of electrostatic fields and glycosphingolipid-mediated interfacial polarization on the phosphohydrolytic surface reaction catalyzed by the enzyme. In continuation of our previous studies, this work shows that PLA_2 activity against mixed lipid monolayers of phospholipids and glycosphingolipids can be directly modulated by the application of constant electrostatic fields in relation to their magnitude and polarity. In addition, the effects of the external field are superimposed onto and modulated by the depolarization or hyperpolarization induced at the interface by the specific dipolar properties of the glycosphingolipid molecules in the mixed films.

MATERIALS AND METHODS

Lipid monolayers

Dilauroylphosphatidic acid and dilauroylphosphatidylcholine were from Avanti Polar Lipids Inc. (Alabaster, AL). PLA_2 from porcine pancreas was from Boehringer Mannheim GmbN (Germany). Over 95% of the protein ran as a single band of approx. MW 14,000 on SDS-PAGE. Glycosphingolipids were obtained and purified from bovine brain (31) and gangliosides were additionally purified as reported elsewhere (32). No surface active compounds were detected in the enzyme preparation when injected into the subphase solution, without lipid monolayer, at a concentration 10-fold higher than that routinely used in the assays.

Monolayers of pure dlPA, dlPC, or of their mixtures with glycosphingolipids were prepared as described before (19, 21, 22, 24, 33). Surface pressure- and surface potential-molecular area isotherms of the different lipids and mixtures used reproduced the behavior previously published (3, 30–32). The surface parameters were determined and controlled with a Monofilmmeter with Lift Control (Mayer-Feintechnik, Göttingen, Germany). A specially designed circular Teflon-coated trough was constructed with several compartments whose surfaces are communicated by narrow and shallow slits (1 mm depth × 1 mm wide × 5 mm long) through which diffusion of subphase solution was restricted. Absence of surface active impurities before spreading the monolayers or in the spreading solvents was checked by reducing the initial trough area (18 cm^2, 17 ml subphase volume) to about 10% of the initial area in the absence of spread lipids or by spreading 50 μl of pure solvents; the changes in surface pressure and surface potential were less than ± 1.0 mN/m and ± 30 mV. The lipid monolayers were formed at 30 ± 0.3°C by spreading about 5 nmol lipid in less than 25 μl chloroform–methanol–water 2:1:0.5. After solvent evaporation and monolayer stabilization for 10 min, the lipid monolayers were compressed to the desired initial surface pressure which was thereafter maintained constant (within ± 1.0 mN/m) with the automatic surface barostat of the equipment. The mixed monolayers were stable, with surface pressure-molecular area isotherms reproducible under recompression, and less than 5% leakage occurred during the time of the experiments. This was ascertained by the constancy of the surface pressure and surface area in films over enzyme-free subphases or at surface pressures above the cut-off points for activity.

Film transfer to enzyme-free solutions was performed as described (33) in order to eliminate possible influences of electrostatic fields on pre-catalytic steps of the reaction (such as adsorption and/or interfacial activation of the enzyme) (23, 33). Briefly, the enzyme was injected (100 ng/ml final concentration) into the subphase (buffer: 10 mm Tris/HCl, 20 mm $CaCl_2$, 100 mm NaCl, pH 8.0) under preformed monolayers that were kept at a surface pressure previously determined to be above the cut-off point for activity for each mixed film studied (21, 22). After this the monolayers were transferred onto a different compartment, at the same constant surface pressure, and rinsed over an enzyme-free subphase before bringing them over to the reaction compartment from which the initial subphase containing the excess enzyme was removed, washed, and filled with a solution not containing the enzyme. A reservoir lipid monolayer of the same composition was spread on the surface of the adjacent compartment and adjusted to the same surface pressure with the communicating slit closed. Subsequently the slit was opened (while keeping the surface barostat active to keep the surface pressure constant during this operation); then the surface pressure was rapidly (within 30 sec) brought to the optimum surface pressure for enzyme activity and the reaction started (21). No significant diffusion of adsorbed PLA_2 from the surface of the reaction compartment to the reservoir monolayer occurred during the time of the experiments. This was controlled by closing the slit communicating the compartments at different times, in the presence and absence of applied electrostatic fields (see below), and ascertaining that the surface pressure measured on the side of the reservoir monolayer (**Fig. 1**) remained constant. Unless otherwise stated, the surface pressure was maintained constant at 14 ± 1 mN/m for films containing dlPA or 11 ± 1 mN/m for films with dlPC (21). After the reaction started, electrostatic fields were applied as described below and indicated in Fig. 2. The amount of enzyme protein present in the transferred films before and after the application of the fields was determined after collection of the monolayer with hydrophobic paper (34) as described previously (23).

Electrostatic fields

Electrostatic fields were applied with a constant potential power source and amplifier (World Precision Inst., Sarasota, FL) to an electrode pair across the monolayer interface with an arrangement similar to that described by Thuren et al. (12) and illustrated in Fig. 1. The subphase electrode taken as a reference was a Pt plate covering the whole floor (18 cm^2) of the reaction compartment; the surface electrode was formed by a bronze plate, of exactly the same size and shape as the Pt plate serving as reference electrode, which was covered with a semiconductor silicon rubber sheet (volume resistance 5 ohms-cm) (CHR Ind. New Haven, CT) that was previously treated with octadecylchlorosilane (35). The silanized semi-conductor electrode covering the whole surface of the reaction compartment was positioned horizontally above the monolayer using the pre-

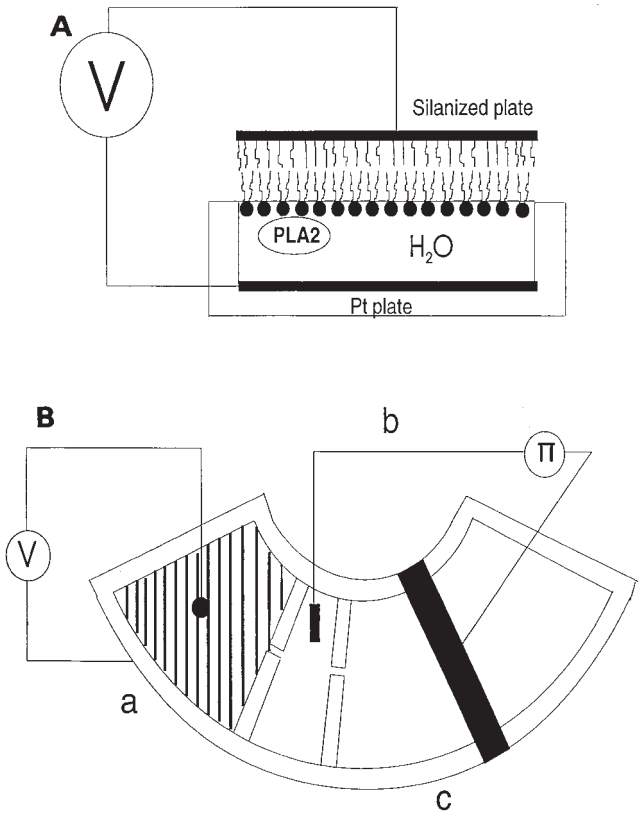

Fig. 1. Simplified illustration of monolayer set-up for determining electrostatic field modulation of PLA_2 activity. Lipid monolayers with adsorbed enzyme above the cut-off surface pressure point for activity were transferred onto an enzyme-free subphase as described in the text. A) Cross-section of the reaction compartment showing the floor Pt plate, serving as reference electrode, and the surface silanized semiconductor electrode positioned in contact with the monolayer (as described in Materials and Methods), and connected to the constant potential power source (V). B) Top view showing the reaction compartment (a) fully covered with the surface electrode (cross-hatched area) and the measuring (b) and reservoir compartments (c) with the surface barostat-operated (π) compression barrier (black area).

cision motor of the Lift Control unit of the monolayer equipment; the electrode was carefully brought to make direct contact with the lipid hydrocarbon chains on the air side of the monolayer surface (35). The surface pressure was maintained constant with the automated surface barostat of the equipment. The monolayer area covered by the electrode was 18 cm^2. Temperature was kept constant at 30° ± 0.3°C with a Haake F3C refrigerated thermocirculating bath and continuously monitored in the monolayer subphase by an immersion thermistor probe connected to a telethermometer. The electrostatic field applied was taken nominally as that indicated by the potential source; the amount of current passing through the monolayer interface (roughly indicative of monolayer integrity) was continuously monitored with a Keithley 610 electrometer, together with the temperature (telethermometer readout), and outputted continuously to a recorder. All the lipid monolayers used exhibited positive surface potentials along the whole surface pressure-molecular area isotherms (3, 36). At the surface pressures used, the surface (dipole) potentials of the different monolayers ranged between 200 and 500 mV (positive on the air side); the positive end of the perpendicular resultant dipole moment vector of the molecules oriented at the interface points toward the hydrocarbon (air) side of the monolayer (28, 29, 36, 37). Because of this, the application of external negative or positive potentials to the surface electrode with respect to the subphase imply a molecular hyperpolarization or depolarization, respectively, of the lipid interface. At the interfacial level, the dipole potential due to the molecules oriented in the monolayer, measured in the absence of external fields, corresponds to electrostatic fields (gradient of the electrostatic potential difference across the molecular length) in the range of 1.5×10^6 to 2.5×10^6 V/cm (28, 29) for the different mixtures used, positive toward the hydrocarbon side. With the trough depth, electrode plates, and system settings used, the maximum external potential applied between the electrode plates corresponds to a potential gradient (electrostatic field) of 100 V/cm. All experiments were done at least four times and the results are expressed as mean ± SEM.

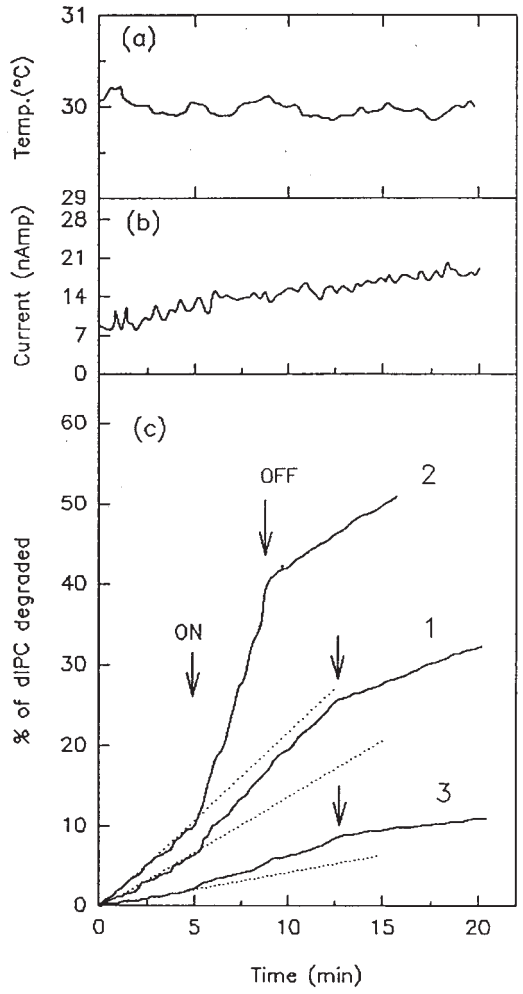

Fig. 2. Reversible activation of phospholipase A_2 activity induced by hyperpolarizing electrostatic fields. The activity of PLA_2 against lipid monolayers at 14 mN/m, as a function of time, is shown (c) for films of pure dlPA (curve 1) and containing 20 mole % of Sulf (curve 2) or ganglioside GD1a (curve 3). Arrows indicate the switching on and off of the electrostatic field (−25 V applied to the surface electrode with respect to the subphase). Dashed lines extending the initial activity after applying the filed are shown to facilitate comparing changes of slope. The upper part shows the variation of temperature (a) or current flowing through the monolayer (b) during the time of the experiment.

RESULTS

Figure 2 represents a typical experiment. In transferred films the phospholipid degradation by PLA_2 starts immediately without a lag-time (Fig. 2) because the pre-catalytic steps of enzyme adsorption and interfacial activation have already taken place (21, 30). Under conditions of continuous supply of fresh substrate from an adjacent monolayer reservoir at the same constant surface pressure, the reaction against the pure phospholipid film proceeds under zero order kinetics (21, 22, 33) because the products formed by PLA_2 activity readily desorb into the subphase solution.

Figure 2 (curve 1) shows the amount of degradation as a function of time. The initial portion of the curves is in the absence of applied fields. The rates of activity in these initial portions are similar to those previously described (21, 22, 33). After 5 min, a potential of −25 V was applied to the upper (silicon semi-conductor) electrode with respect to the suphase and maintained for about 7–8 min before switching it off. The resultant perpendicular dipole moment of the lipids (**Fig. 3** and **Table 1**) point with the positive end toward the air (28, 29, 37); the application of a negative voltage to the surface electrode with respect to the subphase leads to a stretching of the molecular dipole (12) and acts as a hyperpolarizing potential. Immediately after applying the field, the enzyme activity against films of pure dlPA increased to about twice the rate observed in the absence of the field; the change was reversible and the activity returned to the initial rate when the field was switched off. These changes were reproduced in successive cycles. The current passing through the electrodes was essentially constant at a level of between 10–14 nanoamperes during the time of the experiment; after about 1 h and the application of several cycles of electrostatic field, the current increased slowly to about 30 nanoamperes, possibly indicating some deterioration of the monolayer capacity after prolonged time and field exposure. The temperature oscillated within ±0.3°C (Fig. 2a, b). The effects of the electrostatic field on the PLA_2 activity against dIPA are in agreement with previous findings using dlPG as reported by Thuren et al.(12).

Curves 2 and 3 in Fig. 2 correspond to films containing 20 mole % of Sulf or ganglioside GD1a, respectively. The

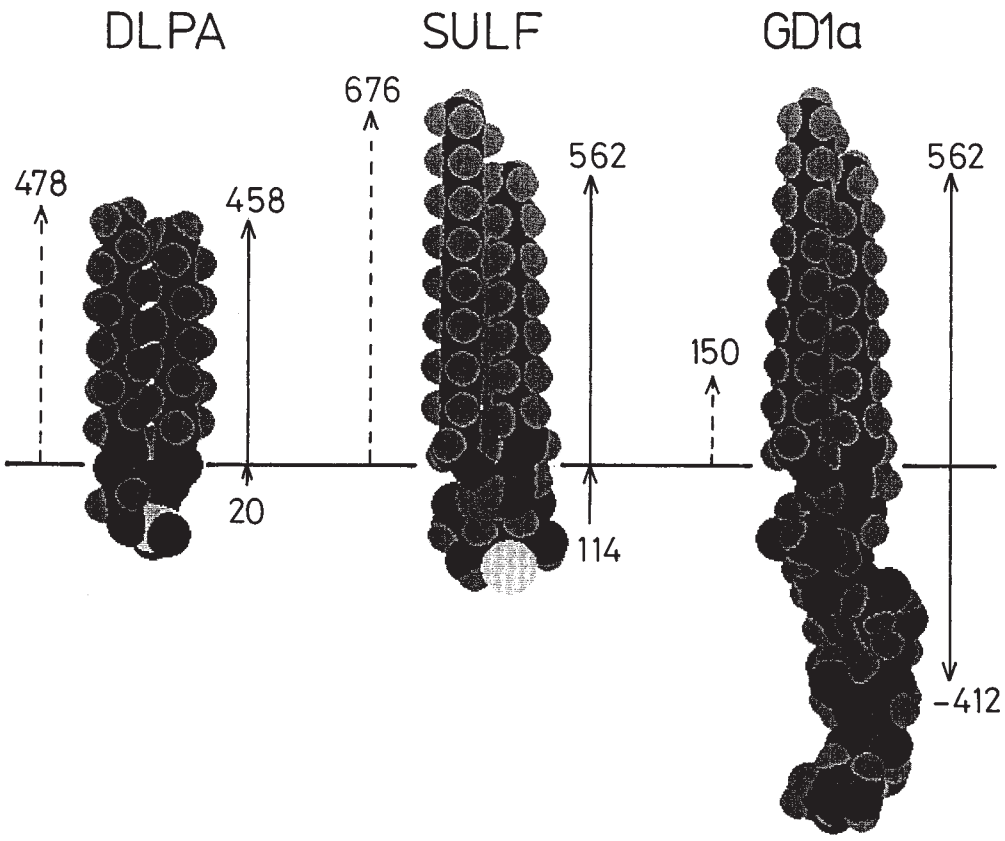

Fig. 3. Molecular models and dipole moments of dilauroylphosphatidic acid, sulfatide and ganglioside GD1a. Molecular models are shown in their optimal conformation after convergence of an energy minimization algorithm for torsion angles, interatomic bond stretching, angle and out-of-plane bending, and van der Waals interactions (Tripos Assoc., St. Louis, MO). The horizontal line indicates the approximate position of the interfacial plane. Dashed arrows represent the resultant perpendicular molecular dipole moments and solid arrows correspond to hydrocarbon and polar head group perpendicular contributions to the resultant molecular dipole moments, measured in milliDebye units (see Table 1). Arrowheads indicate the positive end of the dipoles.

TABLE 1. Resultant dipole moment contributions of dilauroylphosphatidic acid and glycosphingolipids

Lipid	ΔV/n	Molecular Dipole Moment	Polar Head Group Dipole Moment	Hydrocarbon Moiety Dipole Moment
	fV.cm²/molec	*mD*	*mD*	*mD*
dlPA	2.51	478	20	458
Sulf	3.66	676	114	562
GalCer	1.76	469	−93	562
Gg_4Cer	1.54	409	−153	562
GM1	1.43	242	−320	562
Gd1a	1.08	150	−412	562

The surface potential per unit of molecular surface density (ΔV/n), from which the resultant dipole moments in the direction perpendicular to the interface were calculated, was determined from the surface pressure– and surface potential–molecular area isotherms at 15 mN/m. The average overall dipole moment contribution of water molecules in the hydration shell of polar head groups is included in the magnitude of the resultant polar head group dipole moment vector; these components cannot be discriminated from the measurements made (31, 36–38). The dipole moment of the hydrocarbon portion was taken as that obtained experimentally for dilauroyldiglyceride for dlPA and that of natural ceramide for glycosphingolipids (see refs. 31, 36, 38).

rate of activity in the absence of applied electrostatic field reflects the previously known effects of glycosphingolipids: activation of PLA_2 by Sulf and inhibition by the neutral glycosphingolipids and gangliosides (21, 23, 33). The response of enzymatic activity to the applied electrostatic field was different depending on the change previously induced on the phospholipid surface potential by the particular glycosphingolipids present in the monolayer. As shown in Fig. 2, **Fig. 4**, and **Fig. 5**, the electrostatic activation of PLA_2 by a hyperpolarizing potential difference (negative potential to the upper electrode with respect to the subphase) of a same magnitude was enhanced in films containing Sulf and inhibited in those containing GalCer, asialo-GM1, and GD1a (the effects were similar to the latter in films containing GM1) compared to dlPA. Again, the field effect was reversible; the rate returned to the initial level when the field was switched off, and the cycle was reproducible by reapplying the field (Fig. 2). The effects on the PLA_2 activity against dlPC were similar but the response to the applied fields was less than half of those found with dlPA; due to this relatively low sensitivity of the zwitterionic phospholipid, we continued the experiments using mixed films with dlPA. Small variations of surface pressure were observed under the application of the electrostatic fields that were not above 2 mN/m in films with dlPA (increases or decreases with depolarizing or hyperpolarizing fields, respectively). This is also in agreement with the observations by Thuren et al. (12) who suggested that the results probably represent reversible alterations of the intermolecular packing caused by electrostatical stretching-compression of the resultant molecular dipole moment perpendicular to the interface induced by the applied fields. In mixed films with glycosphingolipids, some field-induced oscillations of the surface pressure were observed but these were of small magnitude; because of the limitations of our instrument

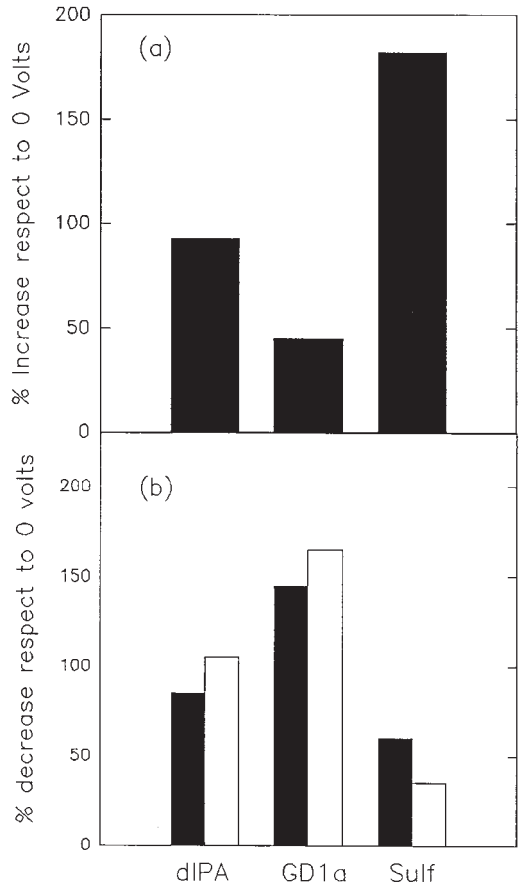

Fig. 4. Comparative changes of phospholipase A_2 activity by hyperpolarizing and depolarizing electrostatic fields. Increase (a) of PLA_2 activity after applying −25 V to the upper electrode with respect to the subphase or decrease of PLA_2 activity (b) after applying +25 V (black bars) or +50 V (white bars) to the upper electrode with respect to the subphase for films of pure dlPA or containing 20 mole % of ganglioside GD1a or Sulf.

to accurately report and reproduce changes of surface pressure below ±1 mN/m, we cannot discuss their significance at present.

In the case of a depolarizing potential of +25 V (positive potential applied to upper electrode with respect to the subphase) the enzymatic activity against films of the pure substrate dlPA is decreased by about 74%. This is in good agreement with the effects reported for dlPG by Thuren et al. (12). Compared to pure films of dlPA, the PLA_2 activity against monolayers containing 20 mole % of GD1a showed a larger decrease when exposed to a same potential difference (Fig. 4b). Conversely, monolayers with Sulf showed a smaller decrease of activity compared to pure films of dlPA.

Figure 5 shows the dependence of the field-induced activation of enzymatic activity on the magnitude of the electrostatic potential difference applied between the surface and subphase electrodes across the monolayer interface. By application of electrostatic potential difference between −5 and −50 V, the results remained qualitatively the same regarding the effects of glycosphingolipids (the presence of Sulf always led to enhanced activity while the

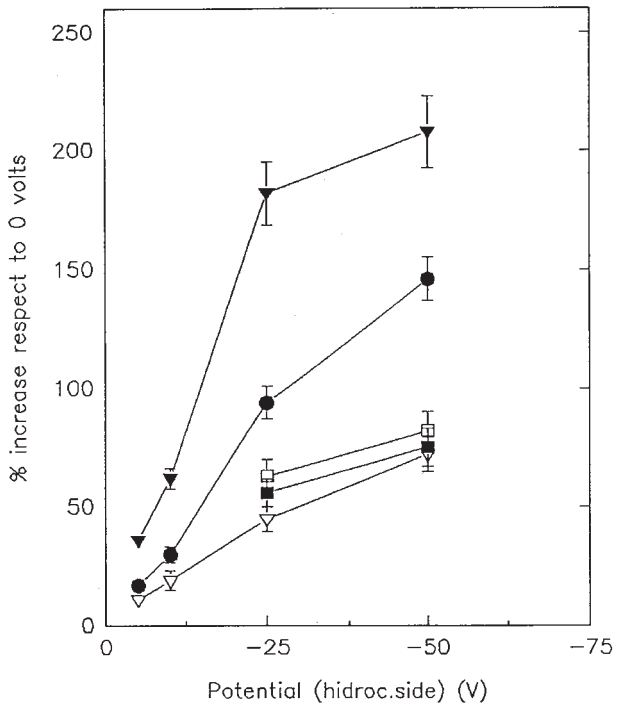

Fig. 5. Dependence of the activation of the phospholipase A_2 activity on the electrostatic field strength. The activity of PLA_2 is shown as a function of the hyperpolarizing voltage applied to the surface electrode with respect to the subphase for films of pure dlPA (●) or containing 20 mole % of Sulf (▼), GD1a (▽), GalCer (□), and asialo-GM1 (■).

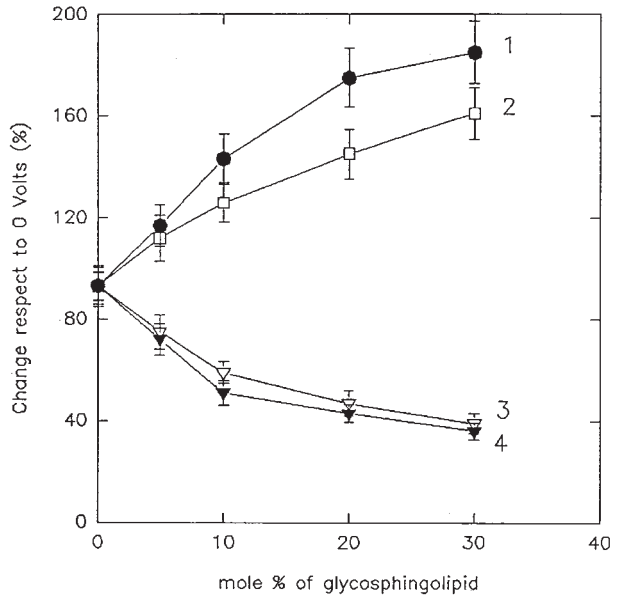

Fig. 6. Dependence of the electrostatic field-induced change of phospholipase activity on the proportion of glycosphingolipid in the mixed monolayer. The percentage change of PLA_2 activity (taken with respect to the activity against the same film in the absence of applied electrostatic field) induced by −25 V (curves 1 and 3) or +25 V (curves 2 and 4) applied to the surface electrode for mixed monolayers of dlPA and Sulf (●, ▼) or GD1a (□, ▽) in the proportions indicated in the abscissa. The point on the ordinate corresponding to 0 mole % glycosphingolipid represents the change induced by the applied field on the PLA_2 activity against films of pure dlPA.

presence of the neutral glycosphingolipids and ganglioside decreased the rate of phospholipid degradation for each hyperpolarizing electrostatic field applied). Figure 5 also indicates that in order to obtain a similar electrostatically induced percentage change of activity in films containing glycosphingolipids compared to pure films of dlPA, it is necessary to apply a larger hyperpolarizing potential difference across the interface when the films contain neutral glycosphingolipids or ganglioside, while a smaller potential is required for mixed monolayer with Sulf. The application of depolarizing electrostatic fields across the monolayer led to the opposite conclusion (not shown). In agreement with previous studies (23) the amount of enzyme protein associated to the transferred films was similar for all mixtures (0.4–0.5 pmol/cm^2) and no detectable variations of this amount occurred by application of the fields.

Figure 6 shows the % change of PLA_2 activity induced by hyperpolarizing or depolarizing fields acting on films containing different proportions of GD1a or Sulf. The effects of the applied fields can be detected in monolayers containing relatively small proportions of glycosphingolipids (<10 mole %). This indicates that any local perturbation induced by the dipolar properties of glycosphingolipids is spread out to a long range over the whole monolayer surface. This is in keeping with early studies (3, 28, 29, 31) showing that the pair intermolecular interaction energies in these films are in the range corresponding to delocalized surfaces on thermodynamic terms.

The mean molecular dielectric polarization vector (P) of the oriented lipids represents the resultant perpendicular dipole moment per unit volume at the interface (39) and it is expressed as units of charge per unit area (coulomb/m^2). In terms of physical units (equivalent to joule/volt·m^2) this parameter is also a representation of the work involved in ordering the resultant molecular dipole moments in the direction perpendicular to the monolayer surface at the molecular surface density (1/molecular area) determined by the lateral surface pressure. Therefore, the average polarization vector in the mixed films of glycosphingolipids and dlPA at constant surface pressure can be directly calculated from the known surface pressure- and surface potential-area isotherms (3, 24, 28, 29) as the ratio between the surface free energy per molecule (2.γ.A, where A is the mean molecular area at the constant surface pressure used, and γ the surface free energy) and the surface potential per unit of molecular surface density (ΔV. A where ΔV is the surface potential and A is again the mean molecular area). **Figure 7** represents the variation of the changes of PLA_2 activity induced by applied hyperpolarizing or depolarizing electrostatic field as a function of the mean resultant molecular polarization vector of mixed monolayers of dlPA with glycosphingolipids. The presence of Sulf causes a decrease of P (24) compared to pure films of dlPA (this reflects an increase of positive electrostatic potential on the hydrocarbon side of the interface compared to the intrinsic polarization of the

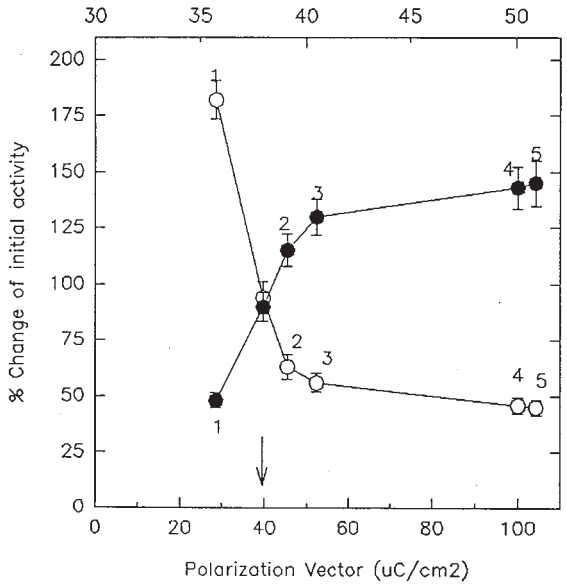

Fig. 7. Variation of PLA_2 activity induced by applied external electrostatic fields as a function of the mean polarization vector in mixed monolayers. The effect on PLA_2 activity on mixed films of dlPA with 20 mole % of Sulf (1), GalCer (2), Gg4Cer (3), GM1 (4), or GD1a (5) is shown after application of a hyperpolarizing potential of −25 V (○) or a depolarizing potential of +25 V (●) to the upper electrode. The arrow indicates the activity and polarization vector in a pure film of dlPA. The figures on the lower abscissa represent the weighted dielectric polarization vectors in the mixed films, calculated according to the individual polarization vectors of the lipids and their respective proportions in the monolayer (20 mole % glycosphingolipid); the figures on the upper abscissa represent the dielectric polarization vectors of the individual lipids in monolayers at the same surface pressure.

pure substrate) and this induces enhanced enzyme activation by the external hyperpolarizing potential applied to the upper electrode. This is in keeping with the lipid interface being already hyperpolarized by Sulf and thus leading to an enhanced response to the applied field compared to pure dlPA. Conversely, in mixed monolayers in which the glycosphingolipid has induced an increase of P compared to that of pure dlPA (reflecting a decrease of the positive electrostatic potential on the hydrocrocarbon side of the interface), the increase of PLA_2 activity induced by the same potential gradient was less. Again, this is in keeping with the substrate being partially depolarized initially by these glycosphingolipids thus leading to a diminished response to an external hyperpolarizing field, while the opposite effect was found by application of depolarizing electrostatic potentials to the upper electrode. Figure 7 also indicates that the changes induced by the external fields on the PLA_2 activity are especially sensitive to small variations of the mean molecular dielectric polarization vector in the range of values corresponding to that of the pure substrate.

DISCUSSION

We previously showed that the activation or inhibition of PLA_2 activity by glycosphingolipids was exerted directly at the interfacial level, most probably through changes induced by modification of the substrate intermolecular organization (21, 23, 33). We also observed that the effects on the enzymatic activity could be significantly correlated to the local electrostatic properties (expressed by the specific dielectric polarization vector) of several natural and semi-synthetic sphingolipids (24).

A detailed mechanistic interpretation of the present results cannot yet be made. However, it is possible to discuss them in the context of previously published work in order to suggest possible logical explanations. The contributions of the hydrocarbon portion and polar head group moieties to the surface electrostatics in ganglioside and chemically related glycosphingolipids have been systematically described previously (3, 28, 29, 31). In neutral glycosphingolipids and gangliosides, the resultant overall component of the polar head group dipoles (in the direction perpendicular to the interface) points towards the aqueous subphase (Fig. 3 and Table 1). This is opposite to the resultant perpendicular dipole moment contributed by the hydrocarbon chains (3, 28, 29, 31, 37). In essence, this signifies the establishment of a local electrostatic potential by the oligosaccharide chain of these glycosphingolipids that effectively reduces the resultant polarity of the lipids at the interface depending on the complexity of the polar head group (3, 28, 37). As a consequence, the net overall perpendicular molecular dipole moment of the lipid monolayer, the parameter that is directly proportional to and is responsible for the surface electrostatic potential across the molecular length (38), is decreased in films with different proportions of GalCer, asialo-GM1, and ganglioside by a proportion that is, in each case, determined by the number and type of carbohydrate residues (3, 28, 29). On the other hand, the negative charge in the polar head group of Sulf is located below the carbohydrate moiety and positioned toward the aqueous side with respect to the interfacial plane (Fig. 3); this determines that, different from galactocerebroside, the contribution of the polar head group dipole moment vector is oriented in the same direction as that of the hydrocarbon chains (3, 30) (Table 1). This leads to an effective interfacial hyperpolarization of phospholipid monolayers containing Sulf. Our results with externally applied electrostatic fields show that, compared to films of pure dlPA, a less intense hyperpolarizing field is required to activate the enzyme when the interface is already hyperpolarized by the presence of Sulf. Conversely, the hyperpolarizing field needs to be more intense in order to increase the rate of PLA_2 activity against monolayers that are initially depolarized locally by the presence of neutral glycosphingolipids and gangliosides. The reverse occurs by the application of depolarizing electrostatic fields (see Figs. 4 and 5).

A recent study using electron paramagnetic resonance (40) investigated the position of bee venom PLA_2 on the membrane surface of a phospholipid analogue. These results provided evidence showing that this enzyme is not deeply inserted into the phospholipid matrix but probably attaches to the interface by the interfacial recognition

site (41) on one end of the molecule while the other end remains flexible and fluctuating oriented toward the aqueous millieu (40). The precise location and positioning of porcine pancreatic PLA_2 at a phospholipid interface is not known. However, our observations on the effects of glycosphingolipids and electrostatic fields on the activity of this enzyme against lipid monolayers would be consistent if we assume that it may be positioned similar to the bee venom enzyme. We reported previously that the adsorption of PLA_2 to the phospholipid interface induced a rapid and reproducible decrease of the surface potential in proportion to the amount of enzyme adsorbed (20). This change occurred during the time period corresponding to the lag-time period during which PLA_2 surface activation takes place, and the surface potential remained constant thereafter and throughout the steady state catalytic reaction (20). The decrease of surface potential caused by the adsorbed protein reflects the presence of an overall resultant dipole moment contribution to the interfacial molecular dipoles that is opposite in direction to that of the phospholipid substrate; this means that the enzyme, consistent with its cationic nature, contributes with a resultant dipole moment along the perpendicular to the interface that bears a positive end oriented toward the aqueous subphase. The application of an electrostatic potential difference negative on the lipid hydrocarbon side across the monolayer should enhance activity by driving the loose end of the adsorbed enzyme molecule toward the interface and bring about a closer interfacial attachment. This should facilitate penetration of a phospholipid molecule into the hydrophobic cavity of the enzyme active site (40, 41). The opposite should be expected, as observed, by application of an electrostatic field of the reverse polarity. Although more work certainly needs to be done to investigate these possibilities, the experimental data that is described above would be highly consistent with the effects observed by Thuren et al. (12) and in our own experiments; the latter also indicated clearly that the local resultant molecular dipoles of the glycosphingolipids can modulate the PLA_2 activity on their own, based on similar explanations.

Our results are consistent with reports showing that phospholipid polar head groups can act as surface electrometers and sensors of the interfacial electrostatic field (42, 43). The phospholipid polar head group dipole is positioned, on average, parallel to the interface and with the positive end of the average polar head group dipole pointing away from the phosphate ester linkages (42). The application of a negative potential to the hydrocarbon side of the interface with respect to the aqueous subphase should stretch the molecule and bend the phospholipid polar head group dipole toward the hydrocarbon phase (44). This should favor a better exposure and coordination of the acyl ester bonds to the active site cavity of the adsorbed enzyme for cleavage (41). The reverse would be expected by the application of a positive potential difference.

The change of activity induced by the applied external fields is maximum for electrostatic potentials between -10 and -25 V as indicated by the increased slopes of the lines in Fig. 5 (this also occurs for positive potentials applied to the upper electrode as summarized in Fig. 4). Within the context discussed above, this suggests that electrostatic fields of that magnitude may represent thresholds for maximum alterations of the lipid molecular dipoles or for the positioning of the adsorbed enzyme. However, the reality is bound to be more complex and will most likely involve effects at various simultaneous levels. It was shown earlier (21) that in the absence of applied fields, the activity of PLA_2 against films of dlPA with 20 mole % Sulf or GD1a was changed, on average, by 130% and -70%, respectively, compared to that against pure dlPA monolayers (21). For example, we found that the application of -25 V to the upper electrode causes a 90% increase of enzymatic activity in films of pure dlPA compared to that found in the absence of applied fields. If both the effects of the glycosphingolipids and the applied field were merely additive, the changes of PLA_2 activity should have been close to about 220% for films containing Sulf and 20% for monolayers with GD1a which were not quite the figures observed (Fig. 4). Most probably, some reorientation of the glycosphingolipid dipoles also occurs under the influence of the field but the effects of these combined effects cannot be separated with the results obtained so far.

In addition, one cannot disregard electrostatically induced effects on the very mechanism of catalysis and it is interesting to consider how our results would compare to what is known about the postulated intrinsic mechanisms of the phosphohydrolytic reaction catalyzed by secretory class I/II PLA_2s. Ca^{2+} ions are essential for unifying enzyme binding to the phospholipid substrate and catalysis; the bivalent cation is responsible for oxygen-mediated binding of the *sn*-3 phosphate group and for the formation and localization of the nucleophilic attack on the *sn*-2 carbonyl of the ester linkage, with electrophilic stabilization of the tetrahedral intermediate (41, 45). In the absence of applied electrostatic fields, Ca^{2+} interacts with lipid polar head groups in monolayers of zwitterionic and anionic phospholipids and their mixtures with glycosphingolipids, as indicated by concentration-dependent changes of the surface (dipole) potential which may be accompanied by alteration of molecular packing depending on the condensed or expanded state of the monolayer (46, 47). The increase of surface potential induced by Ca^{2+} indicates that the ion contributes with an additional resultant dipole with a positive end pointing toward the air side of the monolayer interface; this suggested that the cation is located in the aqueous region in a plane displaced above that of the phospholipid phosphate group and toward the hydrocarbon side (46–48). The application of a negative potential to the surface electrode with respect to the reference in the subphase (or local lipid hyperpolarization) could drive Ca^{2+} to a facilitated position for phosphate binding and the consequent nucleophilic attack to the *sn*-2 carbonyl. The opposite effect should be expected with positive potential differences (or lipid depolarization). On the other hand, apart from possible effects of surface

electrostatics on the productive interfacial attachment of PLA_2 considered above, the ability to initiate the catalytic hydrolysis of the phospholipid may be affected by local and applied electrostatic fields through an effect on the abstraction of a proton by the His48 residue in the active site of the enzyme (41, 45). X-ray diffraction studies position this residue above the plane of the region corresponding to the interfacial glycerol backbone of the phospholipid (41, 49). As delocalized electrons in aromatic carbon rings are readily polarizable, the ability to abstract a proton from the water molecule bound to the Nδ1 atom of His48 (41, 45) should be enhanced by surface hyperpolarization and impaired by depolarization. In some secretory phospholipases of class I/II there is a nearby secondary Ca^{2+} ion acting as a supplemental electrophile which hyperpolarizes the peptide focusing its positive charge directly on the oxyanion (41), while in other enzymes such as bovine pancreatic PLA_2, a similar effect appears to be accomplished by the Lys22 residue (41, 49) which also hyperpolarizes the peptide. Thus, whichever the detailed mechanism, hyperpolarization of the interface is consistent with a facilitation of catalysis by several effects while the reverse should be expected by depolarization.

In summary, the effects of applied external electrostatic fields and the local perturbations induced by the local polar head group properties of glycosphingolipids on their own or in combination with the external fields appear suggestively consistent with a synergistic or concomitant polarity-driven modification of the interfacial positioning of the adsorbed enzyme, reorientation of the lipid polar head groups in the monolayer, and alterations of the intrinsic catalytic mechanism. The effects described indicate the existence of further levels of biophysical control, apart from the changes in molecular packing or phase state (3), formation of non-bilayer phases (25, 27), and overall topology of the lipid aggregate (26), by which glycosphingolipids can participate as effective biomodulators of membrane-mediated events (3). The electrostatic modulation of phosphohydrolytic activity by non-substrate lipids with specific dipolar properties, and in relation to their proportion in the interface, may be important for the long-range regulation of membrane-mediated transduction of information and enzymatically mediated cross-talk between different phosphohydrolytic pathways at the lipid interface (19).

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