Modulation of phospholipase A₂ activity by neutral and anionic glycosphingolipids in monolayers

Ismael D. BIANCO, Gerardo D. FIDELIO and Bruno MAGGIO*

Departamento de Quimica Biologica–CIQUIBIC, Facultad de Ciencias Quimicas–CONICET, Universidad Nacional de Cordoba, Casilla de Correo 61, 5016 Cordoba, Argentina

The effect of neutral (galactocerebroside and asialo-ganglioside G_{M1}) or anionic (sulphatide and gangliosides G_{M1}, G_{D1a} and G_{T1b}) glycosphingolipids on the activity of phospholipase A₂ from pig pancreas was studied in mixed monolayers of dilauroyl phosphatidylcholine with the glycosphingolipids in different molar fractions at various constant surface pressures. The activity of the enzyme depends on the proportion and type of glycosphingolipid in the interface. Sulphatide activates the enzyme at all proportions, whereas galactocerebroside shows inhibition or activation depending on its proportion in the film. Asialo-ganglioside G_{M1} and gangliosides G_{M1}, G_{D1a} and G_{T1b} can strongly inhibit the enzyme at relatively low molar fractions in the film in the following order: asialo-ganglioside G_{M1} < ganglioside G_{M1} < ganglioside G_{T1b} < ganglioside G_{D1a}. The changes of activity are not due to a direct action of the lipids on the active centre or interfacial recognition region of the enzyme.

INTRODUCTION

Phospholipases of the A₂ type are ubiquitous enzymes that occur in either extra- or intra-cellular compartments. These enzymes participate in several key events such as the metabolism of phospholipids, turnover of membranes and the formation of bioactive compounds that may act as signal transducers for membrane-mediated phenomena. Natural or synthetic surface-active compounds and the presence of other lipids as well as the composition, intermolecular organization and physical state of the surface have profound influence on the activity of phospholipase A₂ (Van den Bosch, 1980; Volwerk & de Haas, 1982; Dennis, 1983; Verger & Pieroni, 1986).

Phospholipase activities have been implicated in phenomena related to membrane fusion (Lucy, 1978; Hawthorne & Pickard, 1979), modifications of the membrane potential and release of neurotransmitters by exocytosis (Sen & Cooper, 1978). A similar mechanism has been postulated for the effect of β -bungarotoxin, which contains an associated phospholipase A₂ activity (Wernicke *et al.*, 1975). As far as we know, no study on the possible influence of GSLs on the activity of phospholipase A₂ has been reported to date. GSLs are enriched in membranes of the central and peripheral nervous system (Ledeen & Yu, 1982) and are capable of inducing membrane instability processes including cell fusion and neurotransmitter release in nerve endings (Maggio *et al.*, 1978a; Monferran *et al.*, 1979; Cumar *et al.*, 1983). The proportion of GSLs in the membrane and the type of oligosaccharide in its polar head group influence the stability, intermolecular arrangement, surface potential, the phase state of the lipid interface and the interaction with soluble or amphipathic proteins (Maggio *et al.*, 1981, 1986, 1987; Fidelio *et al.*, 1982,

1984; Curatolo, 1987). These lipids are thought to mediate a variety of important events for cellular physiology, morphology, development and recognition (Cumar *et al.*, 1970; Fishman & Brady, 1976; Ledeen, 1983; Hakomori, 1986; Curatolo & Neuringer, 1985; Nojima *et al.*, 1988).

In the present work we have studied the activity of PLA₂ from pig pancreas on lipid monolayers constituted by dIPC and containing different proportions of GalCer, Sulph, asialo-G_{M1}, G_{M1}, G_{D1a} or G_{T1b}.

MATERIALS AND METHODS

dIPC and PLA₂ from pig pancreas were from Sigma Chemical Co. The purification and sources of GSLs and the technique, equipment, calculations and reproducibility for measurement of the monolayer properties were described in detail previously (Maggio *et al.*, 1978b). The rates of hydrolysis of dIPC were determined by means of a surface barostat employing a Radiometer pH-stat with a TTT1a titrator unit and a Titrigraph SBR2-C unit fitted to a Beckman LM 600 electronic microbalance as described by Verger & de Haas (1973). The 'zero-order' trough is similar to that described by Rietsch *et al.* (1977) and consists of a reaction compartment (total volume 20 ml; total surface 16.15 cm²) and a reservoir connected through a narrow slit. The lipid spread in the reservoir was dIPC; either for pure dIPC films or in the case of lipid mixtures, this ensures a constancy of composition through an automatic compensation for the film molecules being degraded in the compartment containing the enzyme at constant surface pressure. The surface potential was measured by using a ²⁴¹Am air-ionizing electrode placed 5 mm above the water surface (Maggio *et al.*, 1978b). The trough was enclosed in a thermo-

Abbreviations used: PLA₂, pancreatic phospholipase A₂; GSLs, glycosphingolipids; dIPC, didodecanoyl phosphatidylcholine (dilauroyl phosphatidylcholine); Cer, ceramide, *N*-acylsphingosine; GalCer, Gal β 1-1'-Cer; Sulph, sulphatide, Gal (3-sulphate) β 1-1'-Cer; Gg₂Cer, asialo-G_{M1}, Gal β 1-3GalNAc β 1-4Gal β 1-4Glc β 1-1'-Cer; G_{M1}, Gal β 1-3GalNAc β 1-4Gal(3-2 α NeuAc) β 1-4Glc β 1-1'-Cer; G_{D1a}, NeuAc α 2-3Gal β 1-3GalNAc β 1-4Gal(3-2 α NeuAc) β 1-4Glc β 1-1'-Cer; G_{T1b}, NeuAc α 2-3Gal β 1-3GalNAc β 1-4Gal(3-2 α NeuAc8-2 α NeuAc) β 1-4Glc β 1-1'-Cer.

* To whom correspondence should be addressed.

statically controlled box maintained at $30 \pm 0.5^\circ\text{C}$, and the subphase used (10 mM-Tris/HCl buffer, pH 8, containing 20 mM- CaCl_2 and 100 mM- NaCl) was kept at this temperature with a Haake F3C circulating water bath. The subphase in the reaction compartment was agitated with a magnetic stirrer spinning at about 250 rev./min. Before each experiment the Teflon trough was cleaned with absolute ethanol and rinsed thoroughly with double-distilled water. Each experiment was repeated at least three times and S.E.M. values of the averages were below 7%. In order to compare the enzymic activity in mixtures with different lipid compositions the activity must be normalized by taking into account the dilution of dIPC by the particular proportion of non-substrate lipid and any variations of molecular area due to lipid-lipid interactions. The latter were studied in separate experiments by analysing deviations of the ideal behaviour of molecular areas or surface potential per molecule as a function of the composition of the monolayer as described previously (Maggio *et al.*, 1978c). The normalization can be done in at least three different manners: first, it can be assumed that the intermolecular packing of dIPC is not modified in the mixture and that the effect be fully ascribed to changes of the molecular area of GSLs; secondly, it may be considered that the variations of molecular area distribute proportionally between dIPC and the GSLs, weighted by their respective molar proportions; thirdly, the activity can be normalized by considering that the GSLs do not modify their molecular area and that the total deviation is accounted for by changes in the intermolecular packing of dIPC. Different values for the enzyme activity are obtained depending on the type of normalization, but similar conclusions are reached because the effects of GSLs on the activity of PLA_2 greatly exceed the corrections introduced by the changes in lipid packing due to intermolecular interactions.

RESULTS

Fig. 1(a) is a tracing of an actual recorder output of an experiment showing the kinetics of enzyme activity and the changes of surface potential as a function of time for a film of dIPC kept at a constant surface pressure. The changes of surface potential parallel the variation in time observed for the enzyme velocity; a plateau level represented by a decrease of between 20 and 70 mV is obtained depending on the enzyme concentration or lipid monolayer. The proportionality between the rate constant and the enzyme concentration corresponds to a straight line for every preparation in the conditions employed (Fig. 1b). The variation of surface potential also depends linearly on the enzyme concentration in the subphase (Fig. 1b). The change of surface potential most probably reflects the adsorption of the enzyme on the interface because, as indicated by the zero-order kinetics obtained, the products from dIPC degradation are released into the subphase and continuously replaced by reservoir molecules. Furthermore, these changes occur in correspondence with the lag times, which are usually taken as a measure of the kinetics of penetration and/or adsorption of the enzyme on the interface (Verger *et al.*, 1973; Rietsch *et al.*, 1977). When a film of dIPC is maintained at a surface pressure above $20 \text{ mN} \cdot \text{m}^{-1}$ the lag time, defined as the time interval before reaching a constant enzyme velocity (see Fig. 1a), becomes extremely

Fig. 1. Enzyme velocity and changes of surface potential induced by PLA_2 in monolayers of dIPC

The actual experimental changes of enzyme velocity (—) and surface potential (trace) as a function of time (a) are shown for a film of dIPC at $12 \text{ mN} \cdot \text{m}^{-1}$ and a concentration of $0.025 \mu\text{g}$ of PLA_2/ml in the subphase. The lag time for reaching a constant velocity and the slow decrease in area before the injection of enzyme due to film leakage (·····) are indicated. The slope of the decrease due to leakage (not above 10% decrease in area in 20 min in all cases) is always subtracted from the slope corresponding to dIPC degradation to obtain the enzyme velocity. The dependence of enzyme velocity (○) and changes of surface potential (●) with the concentration of PLA_2 in the subphase (b) are shown for a film of dIPC/Sulph (1:1) at $18 \text{ mN} \cdot \text{m}^{-1}$.

large and no further change of surface potential is detected.

Mixtures of dIPC with GalCer or Sulph exhibit ideal behaviour for the mean molecular areas at the different surface pressures studied, indicating that, on average, the intermolecular packing of the individual lipids is not modified in the mixed film. The presence of Sulph greatly activates PLA_2 , and the activation becomes higher as the proportion of Sulph in the film is increased (Fig. 2). Both the optimum pressure for enzyme activity and the cut-off point are shifted to higher pressures, indicating that, in the presence of Sulph, the enzyme can degrade dIPC above surface pressures at which it can no longer penetrate a pure substrate film (about $20 \text{ mN} \cdot \text{m}^{-1}$). In spite of this activation, the lag time was not significantly modified by Sulph (Table 1).

Fig. 3 shows that GalCer has opposite effects on the activity of PLA_2 depending on its proportion with dIPC:

Fig. 2. Effect of Sulph on PLA₂ activity

The normalized enzyme velocity as a function of surface pressure is shown for monolayers of pure dIPC (Δ) or films of dIPC containing a molar fraction of Sulph of 0.50 ($\blacktriangle$) and 0.75 ($\square$).

Fig. 3. Effect of GalCer on PLA₂ activity

The normalized enzyme velocity as a function of surface pressure is shown for monolayers of pure dIPC (Δ) or films of dIPC containing a molar fraction of GalCer of 0.50 ($\blacktriangle$) and 0.75 ($\square$).

inhibition is found when the molar fraction of GalCer is 0.5, but activation is observed at a molar fraction of 0.75. The cut-off point is not changed but the optimum of surface pressure is displaced from 12 to 15 mN·m⁻¹ and the lag times are not significantly affected. Asialo-G_{M1}, on the other hand, inhibits the PLA₂ activity and lowers the cut-off pressure at all proportions studied (not shown) in a way similar to G_{M1} (see below).

Mixed films of gangliosides with dIPC show marked negative deviations (20–30 %) from the ideal behaviour for mean molecular areas. In this case the normalized values of enzyme velocity are calculated taking into account the deviations from ideal behaviour in addition to the dilution effect (see the Materials and methods section). The effect of G_{M1} is to inhibit strongly the activity of PLA₂ towards dIPC and to shift considerably the cut-off point to lower pressures at relatively small molar fractions (Fig. 4). G_{D1a} or G_{T1b} showed a higher capability for inhibition, and the cut-off pressures moved to lower values in proportion to the film composition.

The enzyme activity was completely abolished at all pressures for molar fractions of 0.5, 0.0625 or 0.125 for G_{M1}, G_{D1a} or G_{T1b} respectively. As shown in Table 1, for films in which strong inhibition is observed and above the cut-off points (i.e. a film of dIPC/G_{M1} at a molar fraction of dIPC of 0.8 at 18 mN·m⁻¹), a difference of the surface potential in keeping with the amount of enzyme in the systems is found (see also Fig. 1b). In mixed monolayers showing inhibition the lag times are similar to those found for pure dIPC (Table 1).

DISCUSSION

The results described clearly show that GSLs can act as modulators of the activity of PLA₂ in organized interfaces. Furthermore, the activity of the enzyme on the mixed films with dIPC depends on the type of GSLs and its proportion in the mixture.

The lag times for the hydrolysis of dIPC in the presence of GSLs are not significantly affected, suggesting that the

Table 1. Kinetic parameters and surface potential changes during PLA₂ activity

The bulk enzyme concentration was 0.025 μ g/ml.

Film constituent	Molar fraction of dIPC	$10^{-14} \times$ Enzyme velocity (molecules/min per cm ²)	Surface pressure (mN·m ⁻¹)	Lag time (min)	Difference in surface potential (mV)
dIPC	1	2.76	12	2.9 ± 0.5	-40
dIPC	1	2.55	15	5.7 ± 0.8	-30
dIPC	1	0.00	22		0
dIPC/Sulf	0.5	4.03	12	2.5 ± 0.5	-30
dIPC/Sulf	0.5	5.41	18	1.9 ± 0.5	-35
dIPC/G _{M1}	0.8	1.70	10	2.9 ± 0.4	-25
dIPC/G _{M1}	0.8	0.00	18		-20

Fig. 4. Effect of ganglioside G_{M1} on PLA_2 activity

The normalized enzyme velocity as a function of surface pressure is shown for monolayers of pure dIPC (Δ) or films of dIPC containing a molar fraction of G_{M1} of 0.20 ($\blacktriangle$), 0.33 ($\square$) and 0.50 ($\bullet$). The double symbols represent the range of variation depending on the normalization employed (see the Materials and methods section).

kinetics of enzyme adsorption on the different interfaces are similar (Table 1). Nevertheless, these intervals should have been different if the effect on activity were derived from a variation of the adsorption process due to mere differences in the free energy of the surface. In addition, even if the magnitude of the enzyme activity could have been modified, the optimum of surface pressure for the maximal activity should have coincided for the interfaces containing different proportions of GSLs. Quite on the contrary, for most of the mixtures the values of optimal surface pressures are changed; furthermore, at a certain proportion GSLs showed activation at some value of surface pressure and inhibition or no change at another or vice versa (note that the curves in Figs. 2–4 are not parallel and even some crossing over occurs). It should be emphasized that the variations of the activity of PLA_2 towards the substrate shown in the present work represent true effects induced by the presence of GSLs in the interface, because the values have already been expressed in a normalized and fully comparative manner by taking into account the surface dilution of dIPC and the deviation of ideal behaviour when applicable (see the Materials and methods section).

When GalCer is present in equimolar amounts to dIPC the enzyme is inhibited, but it is more active when the molar fraction of GalCer is increased in the film (albeit not at all surface pressures). A specific effect of GalCer on the active centre of the enzyme can be ruled out by these results. The effect of Sulph is comparable with that induced in bulk systems by anionic surfactants (Volwerk *et al.*, 1986), and is opposite to the inhibitory effect of dicetyl phosphate in mixed monolayers reported by Hendrickson *et al.* (1981). As shown by Verger *et al.* (1973) and Volwerk *et al.* (1986), the molecular constraints acting in the regulation of the 'interfacial quality' to allow the expression of the enzyme activity are

fairly strict, and small variations in them have amplified consequences on the enzyme. However, the interfaces formed by Sulph or GalCer and dIPC behave as ideal mixtures for either mean molecular area or average surface potential per molecule. This indicates that the marked activation induced by Sulph at all proportions or by GalCer at a molar fraction of 0.75 and the inhibition by the latter at a molar fraction of 0.5 cannot be ascribed to measurable modifications (i.e. more than 5–10%) of the interfacial packing or dipolar organization of dIPC. The sensitivity of the enzyme to detect subtle molecular differences may indeed exceed the possibility for measuring them. Similarly to what was suggested for anionic detergents (Volwerk *et al.*, 1986), Sulph may provide an interface in which the enzyme may have a conformation more favourable for activity; this cannot, however, apply to GalCer at higher molar fractions or to the effect of gangliosides.

Furthermore, the possibility that a negative charge on a non-substrate at the interface determines a unique behaviour (i.e. always either activation or inhibition) of the enzyme activity is not supported by the results obtained with G_{M1} , G_{D1a} or G_{T1b} . In contrast with Sulph, the gangliosides are already inhibitory at relatively low molar fractions and the cut-off pressure is shifted to lower values. The sialic acid moiety is not directly responsible for the inhibition, since asialo- G_{M1} shows a similar pattern of inhibition at comparable molar fractions and surface pressures. In addition, neither free sialic acid or sulphate added in a molar ratio of 100:1 to the enzyme or G_{M1} or Sulph added to the subphase as monomers (below their critical micellar concentration but still in molar ratios over 10:1 with respect to the enzyme) induces any effect on the activity of PLA_2 on a monolayer of pure dIPC at 12 mN·m⁻¹. Therefore the effects induced by GSLs are not due to a direct effect of their chemical groups acting on the active centre or on the interfacial recognition region of the enzyme.

Relatively small differences in the intermolecular arrangement and/or stereochemical orientation of the relevant moieties that may be considered insignificant or are not even detected by deviations from the additivity rule or variations of the phase state of the interface are probably important for regulation of the enzyme activity. It has been reported that lateral defects and coexistence of domains in different phases as well as a preferential adsorption of the enzyme on a particular phase can lead to an enhancement of its activity (Menashe *et al.*, 1981). However, this cannot explain the behaviour of mixtures of dIPC with GalCer at a molar fraction of 0.5 or of mixtures with asialo- G_{M1} or gangliosides because in these conditions the activity of the enzyme is inhibited. Steric effects may be participating to impair the access of the enzyme to the interface, but again no differences in the lag times are observed for the activity of PLA_2 towards interfaces containing gangliosides compared with those of pure dIPC; moreover, the enzyme is capable of perturbing the dipolar properties of the interface, since changes of the surface potential are observed (Table 1). As for Sulph, this lipid is fully miscible (cf. Gaines, 1966; Maggio *et al.*, 1978c) with dIPC in the monolayer as mentioned above. The presence of very small inhomogeneous regions in the film cannot, however, be totally discarded by this methodology.

Changes of electric field across the interface modify the activity of PLA_2 , suggesting that the membrane

potential and electric-field-induced changes in the conformation of the substrate may be important to regulate its activity (Thuren *et al.*, 1984, 1987). Conceivably, changes of the local electric field due to a new dipolar arrangement among the polar head groups depending on the composition and intermolecular packing in the mixed interfaces (Maggio *et al.*, 1980) may be involved. However, in agreement with other data reported for PLA₂ and other phospholipases (Volwerk *et al.*, 1986), our results with GSLs indicate that the pattern of behaviour for PLA₂ cannot be predicted simply on the basis of the electrostatic conditions present at the interface. In becoming part of an organized surface PLA₂ must inevitably cross the energy barrier formed by the polar head groups at the interface and the organized water molecules around them before reaching the acyl ester linkage of the phospholipids. Our results show that the presence of GSLs can either facilitate or interfere with this process in a complex way according to their type and molar proportions, probably through a subtle influence on the intermolecular organization.

This work was supported by grants from CONICOR and CONICET, Argentina. B. M. and G. D. F. are Career Investigators and I. D. B. is a Fellow of the latter institution. We thank Mr. P. Vicente and the Mechanical Precision Workshop, FAMAF, for invaluable help in adapting the 'zero-order' trough.

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Received 31 May 1988/31 August 1988; accepted 20 September 1988