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# Concerted Modulation by Myelin Basic Protein and Sulfatide of the Activity of Phospholipase $A_2$ against Phospholipid Monolayers†

Ismael D. Bianco,‡ Gerardo D. Fidelio,‡ Robert K. Yu,§ and Bruno Maggio*,§

*Departamento de Quimica Biologica-CIQUIBIC, Facultad de Ciencias Quimicas-CONICET, Universidad Nacional de Cordoba, c.c. 61, 5016 Cordoba, Argentina, and Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, MCV Station Box 614, Richmond, Virginia 23298-0614*

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ABSTRACT: The effect of myelin basic protein (MBP) on the activity of phospholipase $A_2$ ($PLA_2$, EC 3.1.1.4) against monolayers of dilauroylphosphatidylcholine (dlPC) or dilauroylphosphatidic acid (dlPA) containing different proportions of sulfatide (Sulf) and galactocerebroside (GalCer) was investigated. MBP was introduced into the interface by direct spreading as an initial constitutive component of the lipid–protein film or by adsorption and penetration from the subphase into the preformed lipid monolayers. The effect of MBP on $PLA_2$ activity depends on the type of phospholipid and on the proportion of MBP at the interface. At a low mole fraction of MBP, homogeneously mixed lipid–protein monolayers are formed, and the $PLA_2$ activity against dlPC is only slightly modified while the degradation of dlPA is markedly inhibited. This is probably due to favorable charge–charge interactions between dlPA and MBP that interfere with the enzyme action. The $PLA_2$ activity against either phospholipid is increased when the mole fraction of MBP exceeds the proportion at which immiscible surface domains are formed. GalCer has little effect on the modulation by MBP of the phospholipase activity. The effect of Sulf depends on its proportions in relation to MBP. The individual effects of both components balance each other, and a finely tuned modulation is regulated by the interactions of MBP with Sulf or with the phospholipid.

In experimental models of demyelination and remyelination and in the pathological lesions of multiple sclerosis and related diseases, there are changes of the content of sulfatide (Sulf),[1] cerebrosides (GalCer), or gangliosides and losses of myelin-specific proteins, particularly myelin basic protein (MBP)

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* Corresponding author.
‡ Universidad Nacional de Cordoba.
§ Medical College of Virginia.

[1] Abbreviations: PLs, phospholipids; GSLs, glycosphingolipids; MBP, myelin basic protein; dlPC (dilauroylphosphatidylcholine), didodecanoyl-*sn*-glycero-3-phosphocholine; dlPA (dilauroylphosphatidic acid), didodecanoyl-*sn*-glycero-3-phosphate; dpPC (dipalmitoylphosphatidyl choline), dihexadecanoyl-*sn*-glycero-3-phosphocholine; $PLA_2$, porcine pancreas phospholipase $A_2$ (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4); PLC, *Clostridium perfringens* phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3); Cer (ceramide), *N*-acylsphingosine; GalCer (galactocerebroside), Gal($\beta$1-1)Cer; Sulf (sulfatide), Gal(3-sulfate)($\beta$1-1)Cer.

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(Einstein et al., 1972; Maggio et al., 1972, 1983; Yu et al., 1974; Maggio & Cumar, 1975; Hirsch, 1981; Yu et al., 1982; Norton & Cammer, 1984a,b; Smith et al., 1984). The alterations are due to the action of enzymes normally present in the central nervous system tissue or that are brought into the inflamed area by immunosensitized cells (Riekkinen & Clausen, 1969; Ledeen, 1984; Smith & Benjamins, 1984; Saito & Yu, 1986; Yohe et al., 1986). These enzymes include proteinases and phospholipases, all of which initiate or enhance myelin and nerve cell disruption (Smith & Benjamins, 1984). The intermolecular organization of glycosphingolipids, phospholipids, and MBP and the relative amounts in the myelin membrane may regulate the possibility for their degradation by exogenous enzymes. Myelin is much more active than was originally thought, both on a metabolic and a structural basis (Larocca et al., 1987; Kahn & Morell, 1988; Moscarello, 1989). In experimental demyelinating diseases, early changes of the myelin organization have been observed in direct connection with cytoplasmic processes of immunosensitized cells that infiltrate among the myelin lamellae causing their separation and the occurrence of membrane fusion, recombination, and vesiculation (Lampe & Kies, 1967; Dal Canto et al., 1975). It is important to understand at the molecular level how different initial surface conditions can regulate the vulnerability of the membrane and the interactions responsible for regulating the degradation of myelin constituents. An inherent difficulty when employing natural membrane preparations is the lack of control of the various molecular factors that participate in these events. The valid alternative is to first describe the major molecular parameters and interactions with model membrane systems under a strict control of the surface parameters. Once these parameters have been identified, their behavior and relevance can then be explored with more complex systems that are progressively more similar to the natural membrane.

The accessibility of a membrane component to exogenous agents depends dramatically on subtle variations of the intermolecular organization (London & Vossemberg, 1973; Boggs et al., 1981; Crook et al., 1986; Verger & Pieroni, 1986; Maggio et al., 1981, 1988a,b). MBP has been localized in the cytoplasmic half of the myelin membrane (Poduslo & Braun, 1975). The location of Sulf is uncertain, but this lipid exhibits marked interactions with MBP and it has been assumed to be associated with it (Demel et al., 1973; London & Vossemberg, 1973). Complexes of MBP with Sulf have been isolated from myelin (Kunishita et al., 1979; Koh et al., 1981). Sulf protects certain regions of the MPB molecule from degradation by trypsin (London & Vossemberg, 1973), while the rate of MBP cleavage by brain cathepsin D is increased by Sulf (Williams et al., 1986). Also, MBP protects gangliosides such as $G_{M4}$ that are enriched in myelin from neuraminidase attack (Yohe et al., 1983). In addition, MBP is capable of modifying the intermolecular organization, stability, permeability, recombination, and topology of bilayer membranes formed by PLs and the above glycosphingolipids (GSLs) (Maggio et al., 1977; Boggs et al., 1977, 1981, 1982; Fidelio et al., 1982, 1984; Maggio & Yu, 1989).

It is well known that the activity of enzymes acting on membrane lipids such as the phospholipases is very sensitive to the molecular interactions, lateral surface pressure, and surface potential of the interface (Verger & Pieroni, 1986; Thuren et al., 1987; Jain & Berg, 1989; Bell & Biltonen, 1989). We have recently shown that changes of intermolecular organization of phospholipids (PLs) induced by the presence of Sulf, GalCer, and several gangliosides affect markedly the activity of phospholipase $A_2$ ($PLA_2$, EC 3.1.1.4) and phospholipase C (PLC, EC 3.1.4.3) (Bianco et al., 1989, 1990, 1991). Various proteins can affect the activity of phospholipases and lipases against monolayers and bilayer membranes (Flower, 1984; Bartolf & Franson, 1987; Gargouri et al., 1987; Conricode & Ochs, 1989). Recently, it has been reported that MBP stimulates a phosphatidylinositol-specific phospholipase C isolated from bovine brain (Tompkins & Moscarello, 1991).

In this work we have investigated the influence of MBP on the activity of $PLA_2$ against mixed monolayers of dlPC or dlPA containing different proportions of Sulf or GalCer in order to better understand the interplay of molecular factors that control the accessibility and degradation of membranes containing Pls and GSLs. It is found that the accessibility of the enzyme to the substrate Pls and its activity are strongly affected by MBP. This depends on the manner in which MBP is associated to the interface, on the lateral surface pressure, and on the relative proportions of GSLs and MBP.

## Experimental Procedures

Dilauroylphosphatidylcholine (dlPC), dilauroylphosphatidic acid (dlPA), and $PLA_2$ from porcine pancreas were from Sigma Chem. Co. Porcine MBP was from Eli Lilly, it was further purified and characterized by SDS–PAGE as described previously (Monferran et al., 1979). GalCer and Sulf were purified as described elsewhere; no contaminating lipids were detected by HPTLC (Maggio et al., 1978, 1987; Monferran et al., 1979). The interactions and miscibility in binary and ternary mixed monolayers of PLs, MBP, and GSLs were evaluated from the analysis of surface pressure- and surface potential-area isotherms (Fidelio et al., 1982, 1984, 1986a,b; Bianco & Maggio, 1989). The equipment, technique, and reproducibility for measuring the surface parameters and the assay of phospholipase activity against mixed monolayers were described in detail previously (Maggio et al., 1978; Bianco et al., 1989, 1990, 1991; Bianco & Maggio, 1989). The aqueous subphase consisted of 10 mM Tris-HCl, 20 mM $CaCl_2$, and 100 mM NaCl, at pH 8.0. The experiments were performed in duplicate or triplicate at 30 ± 1 °C. The reproducibility was better than ±5% for the enzyme velocities, ±0.3 $nm^2$ for the mean molecular area, ±10 mV for the surface potential, and ±1 $mN \cdot m^{-1}$ for the surface pressure. Short-chain phospholipids were selected as substrates because the reaction products of the enzymatic reaction are immediately desorbed from the monolayer into the subphase. In this manner, a constant composition and molecular packing can be maintained at the surface; this results in zero-order kinetics (Verger & Pieroni, 1986; Bianco et al., 1989, 1990).

The effect of MBP, present as an initial component of the mixed monolayer, on the $PLA_2$ activity was studied by directly spreading a mixture of PLs with MBP in the desired proportions (Fidelio et al., 1984). Zero-order kinetics at constant surface pressure (Bianco et al., 1989, 1990) were maintained on the reaction compartment by spreading a monolayer of pure substrate on a reservoir compartment communicating with the first through a narrow and shallow slit (Bianco et al., 1990, 1991). The effect of MBP, initially injected into the subphase and subsequently "adsorbed" or "penetrated" into the interface, was studied after eliminating the excess protein remaining in the aqueous subphase by transferring the film over protein-free subphases as described recently (Bianco et al., 1990, 1991). Briefly, the PLs or PLs–GSLs films were initially set at the desired surface pressure on one of the compartments of a multicompartment trough. A sufficient amount of MBP was injected into the subphase to obtain the maximum surface pressure increase (Fidelio et al., 1982). The surface pressure

was monitored continuously, before and after the protein injection (see also the inset in Figure 2b). Similar to monolayers of pure GSLs or long chain PLs (Fidelio et al., 1982, 1986a), the final equilibrium surface pressure was reached in less than 10 min. After this, the film was transferred, at constant surface pressure, onto another compartment filled with a protein-free subphase and rinsed under stirring. The original subphase in the initial compartment was removed, and the compartment was washed and filled with fresh subphase without MBP. The film was returned, at constant pressure, to this compartment. A monolayer of pure PLs was spread at the surface of the reservoir and compressed to the same surface pressure. The barrier separating the reservoir monolayer and that on the reaction compartment was removed, and the surface pressure was adjusted to the desired value. An appropriate amount of $PLA_2$ was injected into the subphase of the reaction compartment and the time course of hydrolysis was recorded under zero-order kinetics (Bianco et al., 1989). The enzyme velocities against the mixed films were normalized as previously described (Bianco et al., 1989, 1991). This takes into account the dilution of the substrate by the other components at the surface and the variations of mean molecular area due to nonideal lipid–lipid or lipid–protein interactions in the film (Fidelio et al., 1984; Bianco & Maggio, 1989; Bianco et al., 1989, 1990, 1991).

## Results and Discussion

### *(1) Surface Properties of MBP and Interactions with dlPC and dlPA*

Previously, we described the surface properties of MBP and its interactions with long-chain PLs and GSLs in monolayers (Fidelio et al., 1982, 1986b; Monferran et al., 1986). However, the changes of molecular organization induced by MBP in monolayers of short-chain phospholipids have not been published before. These data are necessary to interpret the effect of MBP on the $PLA_2$ activity against dlPC and dlPA and are briefly reported in this section. In the absence of lipids, MBP forms stable monomolecular films with a limiting molecular area of 10.60 $nm^2$ and a collapse pressure of 14 $mN \cdot m^{-1}$ (Fidelio et al., 1984). The spreading properties of MBP allow the study of the behavior of mixed lipid–protein monolayers spread directly at the interface. The surface behavior of mixed films of dlPC or dlPA with MBP is qualitatively similar to that of MBP with GSLs (Fidelio et al., 1982, 1984) and that of the amphipathic basic peptide mellitin with long-chain PLs (Fidelio et al., 1986). When the mole fraction of MBP in a mixed monolayer is less than 0.02 (or less than 0.03 in mixtures with dlPC), the films are monophasic as revealed by a single collapse pressure point (at about 34 and 45 $mN \cdot m^{-1}$ for monolayers with dlPA and dlPC, respectively). These values are within $\pm 2$ $mN \cdot m^{-1}$ of the collapse pressures of the pure lipid monolayers. According to the surface phase rule (Fidelio et al., 1984, 1986a) this indicates that, in these proportions, MBP is homogeneously mixed with the PLs.

At low proportions of protein, the films of dlPC–MBP are ideally mixed and show no significant changes of the mean molecular area or surface potential per molecule of the individual components. On the other hand, in monolayers of dlPA–MBP, the mean molecular area at different surface pressures is that expected for the ideal behavior, but the surface potential per molecule is decreased. This indicates ionic or dipolar interactions between the anionic PLs and the basic protein (Fidelio et al., 1984). When the mole fraction of MBP is 0.05 or higher [in these conditions, at least 55–60% of the film area is occupied by MBP (Fidelio et al., 1984)], the mixed

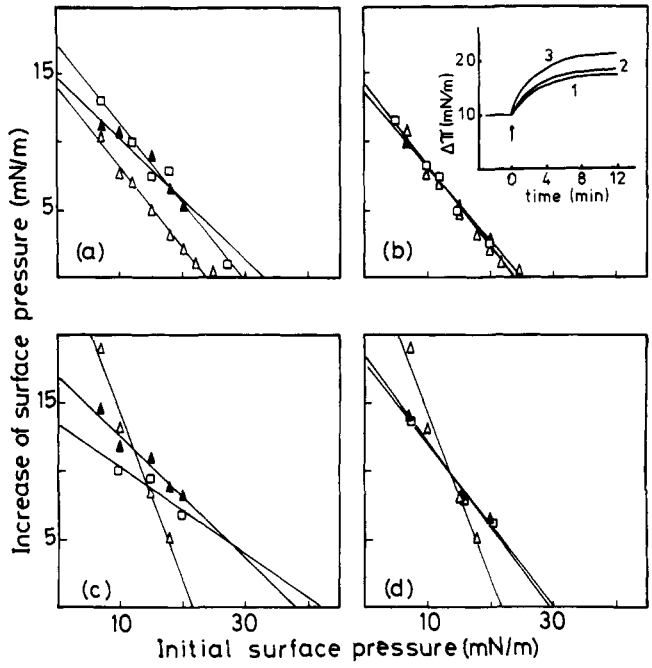


FIGURE 1: Penetration of MBP at different initial surface pressures. Maximal increases in surface pressure after penetration of MBP (100 nM final subphase concentration) into a monolayer of (a) (△) dlPC, (▲) dlPC:Sul (1:1), and (□) dlPC:Sul (3:1); (b) (△) dlPC, (▲) dlPC:GalCer (1:1), and (□) dlPC:GalCer (3:1); (c) (△) dlPA, (▲) dlPA:Sul (1:1), and (□) dlPA:Sul (3:1); (d) (△) dlPA, (▲) dlPA:GalCer (1:1), and (□) dlPA:GalCer (3:1); (inset) time course for the increase of surface pressure (penetration) of MBP into monolayers of dlPC (curve 1), dlPC:Sulf (1:1) (curve 2), and dlPC:GalCer (curve 3).

monolayers with either dlPC or dlPA show biphasic behavior. A surface phase collapsing at about 16–18 $mN \cdot m^{-1}$, together with another showing a collapse pressure at about 44 $mN \cdot m^{-1}$, can be detected. It is likely that the surface phase collapsing at the lower pressure corresponds to a protein-enriched phase immiscible with a lipid-enriched phase that collapses at the higher pressure. This is in keeping with the collapse pressure of the pure protein [above 14 $mN \cdot m^{-1}$ (Fidelio et al., 1982, 1984)] and the increased collapse point (stability) of MBP in the presence of lipids previously described (Fidelio et al., 1984). The presence of these immiscible surface phases depends critically on the composition of the film and on the lateral surface pressure (Fidelio et al., 1982, 1984, 1986a,b). We have found that these factors also have a marked influence on the expression of $PLA_2$ activity (see section 2 below).

When MBP is injected beneath a lipid-free interface, the surface pressure increases in proportion to the protein concentration (Fidelio et al., 1982, 1986a). The maximum value of the equilibrium pressure for adsorption is about 10 $mN \cdot m^{-1}$ at 100 nM MBP in the subphase. The increase of surface pressure ($\Delta\Pi$) obtained by penetration of MBP into monolayers of dlPC or dlPA initially set above 10 $mN \cdot m^{-1}$ is considerable (Figure 1). The increase of surface pressure is a linear decreasing function of the initial pressure (Figure 1). The extrapolation to $\Delta\Pi = 0$ represents the cut-off pressure point above which the protein can no longer penetrate the lipid monolayer (Fidelio et al., 1982, 1986a). The cut-off pressures are in the range of 22–24 $mN \cdot m^{-1}$ for both dlPC and dlPA (Figure 1). The presence of Sulf increases the cut-off for MBP penetration by about 8 $mN \cdot m^{-1}$ for dlPC–Sulf (Figure 1a) and by 18 $mN \cdot m^{-1}$ for dlPA–Sulf (Figure 1c). Similar to the interactions of dpPC with MBP (Fidelio et al., 1982, 1986a), this is due to the considerable stabilization of the protein brought about by the interactions with lipid; this allows MBP

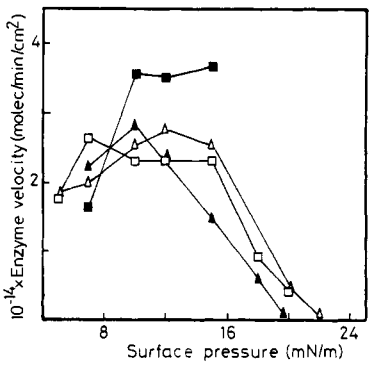


FIGURE 2: Effect of MBP on $PLA_2$ activity. Enzyme velocity as a function of surface pressure for monolayers of pure dlPC (△) or dlPC with a mole fraction of MBP of 0.01 (▲), 0.03 (□), and 0.05 (■). The subphase contained a final concentration of $PLA_2$ of 0.025 μg/mL. The other conditions were as described under Experimental Procedures. The values shown are the average of duplicate or triplicate experiments. The SEM were below ±5% of the average values.

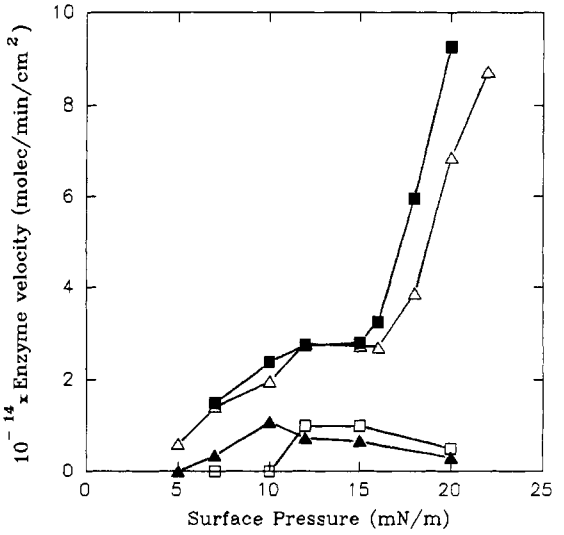


FIGURE 3: Effect of MBP on $PLA_2$ activity. Enzyme velocity as a function of surface pressure for monolayers of pure dlPA (△) or dlPA with a mole fraction of MBP of 0.01 (▲), 0.02 (□), and 0.05 (■). Other conditions and the reproducibility are as described in Figure 2.

to support a much higher lateral pressure without collapsing. By contrast, GalCer causes no change of the cut-off pressure in films with dlPC (Figure 1b) and induces a smaller increase of the cut-off point than Sulf in monolayers with dlPA (Figure 1d). These results are in keeping with the more favorable interactions of MBP with negatively charged compared to neutral GSLs (Fidelio et al., 1982, 1984).

*(2) $PLA_2$ Activity against Lipid–MBP Monolayers*

*(a) Effect of MBP Spread as an Initial Component of the Interface.* Figure 2 shows the rate of hydrolysis of pure dlPC and of mixed monolayers with MBP as a function of the surface pressure. The optimal surface pressure for maximal activity of $PLA_2$ is broad, and it is not markedly modified by MBP up to a mole fraction of 0.03, compared to that found against pure dlPC. However, at a mole fraction of MBP of 0.05, a clear increase of the enzyme velocity is observed in the range of 10–16 mN·m$^{-1}$ (Figure 2). At this proportion of MBP (or above, not shown) and near 16 mN·m$^{-1}$, the films become biphasic (see previous section). It is likely that the enhanced activity is due to the enzyme already detecting some degree of film inhomogeneity as this range of pressure is approached; the extreme sensitivity of $PLA_2$ to detect regions of lateral defects is well known (Menashe et al., 1981; Jain & Berg, 1989; Grainger et al., 1980).

Different from that with dlPC, the reaction of $PLA_2$ against pure dlPA increases with the surface pressure (Figure 3) (Bianco et al., 1990). This is again in keeping with an increase of the enzyme activity in biphasic regions since dlPA shows a liquid-expanded to a liquid-condensed phase transition above 18 mN·m$^{-1}$ (Bianco & Maggio, 1989). With dlPA, MBP below a mole fractions of 0.05 induces marked inhibition of the enzyme (Figure 3). At a mole fraction of 0.05 or above, the original activity is either restored or increased, depending on the surface pressure. When the mole fraction of MBP is below 0.02, dlPA and MBP form a homogeneously mixed monolayer in which the surface potential per molecule is decreased compared to ideally mixed films (see previous section). This is probably due to favorable charge–dipole interactions of dlPA with the basic protein. Experiments with solvatochromic probes that reside at the interface have shown that MBP can change the more polar interfacial environment induced by acidic PLs and GSLs to values of lower micropolarity comparable to those of neutral lipids (Montich et al., 1985, 1988). The favorable interactions between MBP and dlPA can explain the lower activity of $PLA_2$ on the basis of a decreased effective substrate availability. However, the latency time [interval before reaching constant enzyme velocity; see Verger and Pieroni (1986), and Bianco et al. (1989, 1990, 1991)] are not significantly modified in the presence of MBP, and this suggests that the precatalytic steps (adsorption, penetration, and activation) are, on average, similar for the different interfaces (Bianco et al., 1990, 1991). Thus, homogeneously mixed films of the phospholipids with MBP lead to different effects on $PLA_2$ activity depending on the phospholipid substrate: almost no effect on the hydrolysis of dlPC while that of dlPA is inhibited.

When the mole fraction of MBP is 0.05 or higher, the mixed films become biphasic as the surface pressure is increased (see previous section). The resultant increase of enzyme activity against dlPC and dlPA at this mole fraction of MBP probably results from film inhomogeneity in the mixed films; lateral defects are known to enhance $PLA_2$ activity (Upreti & Jain, 1980; Menashe et al., 1981; Grainger et al., 1990). MBP interacts preferentially with acidic PLs (Boggs et al., 1977), and, in mixed systems with GSLs, the protein induces formation of phase-separated domains enriched in phosphatidylcholine (Maggio et al., 1987).

Figure 4a shows the effect of the simultaneous presence of MBP and Sulf in mixed monolayers with dlPC. Enhancement of $PLA_2$ activity is observed at mole fractions of Sulf below 0.35, and the maximal enzyme velocity is displaced toward higher values of surface pressure. The velocity at lower pressures is also higher when the mole fraction of MBP is increased. This may be because the favorable interactions of MBP with Sulf (Fidelio et al., 1982, 1984; Monferran et al., 1986) induce an increase of inhomogeneity at the surface. Decreases of the average surface potential per molecule are found in films containing Sulf and MBP due to dipolar interactions (Fidelio et al., 1984; Monferran et al., 1986). Also, it has been shown that MBP markedly affects the thermotropic behavior of Sulf and induces phase separation of enriched phospholipid domains in mixtures of Sulf with dpPC at mole fractions of MBP below 0.01 (Maggio et al., 1987). Mixed monolayers constituted by dlPC, GalCer and MBP show increased enzyme activity at surface pressures between 10 and 15 mN·m$^{-1}$, but the optimum of enzyme activity is not changed (Figure 4b). The latency times for the enzyme action in all

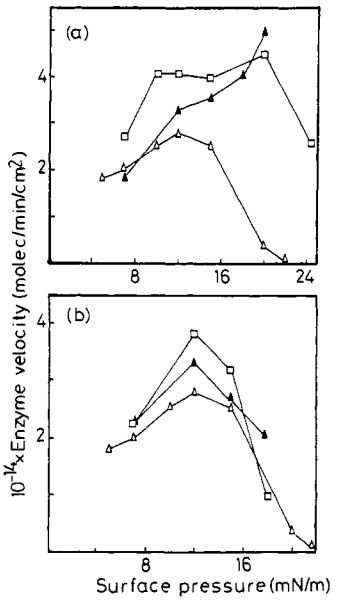


FIGURE 4: Effect of MBP and Sulf (a) or MBP and GalCer (b) on $PLA_2$ activity. Enzyme velocity as a function of surface pressure for monolayers of (a) pure dlPC (△) or ternary films of dlPC:Sulf:MBP in molar ratio of 74:25:1 (▲) and 73:24:3 (□); (b) pure dlPC (△) or ternary films of dlPC:GalCer:MBP in molar ratio of 74:25:1 (▲) and 73:24:3 (□). The enzyme velocity of mixed monolayers in the same lipid proportions but without MBP was within ±10% of the activity against pure films of dlPC at the same surface pressures. At molar fractions of Sulf or GalCer ≥0.5, the enzyme activity was, respectively, activated or inhibited (Bianco et al., 1989, 1990). The reproducibility of the determinations is as stated for Figure 2.

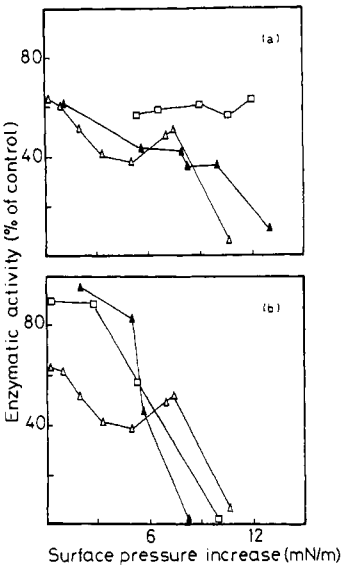


FIGURE 5: Effect of "penetrated" or "adsorbed" MBP on $PLA_2$ activity. The enzyme velocity is expressed as a percentage of the values obtained against a monolayer of pure lipid (100%), as a function of the increase in surface pressure caused by MBP: (a) dlPC (△) or dlPC with a mole fraction of Sulf of 0.25 (▲) or 0.5 (□); (b) dlPC (△) or dlPC with a mole fraction of GalCer of 0.25 (▲) or 0.5 (□). See the text for details. The reproducibility of the determinations is as stated for Figure 2.

these films are not significantly modified. This suggests that the effects on the enzyme activity are exerted at the interface, beyond the initial step of adsorption (Bianco et al., 1989, 1990, 1991). Previous work showed lesser interaction of MBP with GalCer than with Sulf (Demel et al., 1973; Fidelio et al., 1982; Maggio et al., 1987). However, the thermotropic behavior of GalCer is affected by MBP proportionally to the amount of protein; a proportion of the lipid has been perturbed by the protein and no longer participates with the same degree of cooperativity in the phase transition (Maggio et al., 1987). The increased activity of the enzyme is in keeping with the existence of inhomogeneous regions.

(*b*) *Effect of Adsorption and Penetration of MBP from the Subphase.* The lipid monolayers were set at different initial surface pressures to allow a different penetration of MBP (see Figure 1). The protein was injected into the subphase to give a final concentration of 100 nM and left enough time, under stirring, to reach the plateau level for the increase of surface pressure (see the inset in Figure 1b). After reaching the plateau level, the excess protein in the subphase was eliminated by film transfer and rinsing, at constant surface pressure (plateau level) as described under Experimental Procedures. The constancy of the surface pressure was continuously monitored during the film transfer to control possible protein desorption; in all cases the pressure loss was below 10%. After transfer, the lipid–protein monolayer was adjusted to 15 $mN \cdot m^{-1}$, and the zero-order kinetics of $PLA_2$ activity were recorded. In this manner, we could compare the enzyme activity against monolayers that differ in the degree of initial interaction with MBP from the subphase, as measured by the increase of surface pressure (Figure 5). The increase of surface pressure is proportional to the interaction and penetration of MBP into the interface (Demel et al., 1973; Fidelio et al., 1982, 1986a), and in our experiments the excess protein in the subphase was eliminated. The results are expressed as a percentage of the enzyme activity against a monolayer of the corresponding mixed monolayer of lipids but without MBP which were submitted to the same protocol.

Figure 5a shows the relative rates of hydrolysis of interfaces formed by pure dlPC or with different proportions of Sulf. With pure dlPC or in films with a low mole fraction of Sulf, there is a progressive inhibition of the enzyme when the penetration of MBP is higher. When the mole fraction of Sulf in the interface is 0.25, a greater increase of surface pressure by MBP is needed (compared to films of dlPC alone) to reach complete inhibition of the enzyme activity. An increase in the proportion of Sulf to a mole fraction of 0.5 or above abolishes the inhibitory effect of MBP. It is important to emphasize the mutual competition occurring between the effects of Sulf and MBP. In the absence of MBP, the enzymatic activity is *increased* by the presence of Sulf at mole fractions of 0.5 or above as thoroughly documented before (Bianco et al., 1989, 1990, 1991). The experiments in Figure 5 show that, at relatively small proportion of Sulf, the activity is *inhibited* by MBP in proportion to its interaction with the film while an increased proportion of Sulf can revert the effect of MBP. This points out the subtle regulation of the molecular interactions that participate in these effects. This may be of considerable biological significance for understanding the regulation of the degradation of these components in the myelin membrane. A similar dual effect of Sulf and MBP that depends on their relative proportions and interactions in the membrane has been previously shown to modulate processes of bilayer apposition, merging, permeability, neurotransmitter release, and cell fusion (Maggio et al., 1973; Monferran et al., 1979; Cumar et al., 1980, 1983; Maggio & Yu, 1989).

It should be emphasized that the values of enzyme activity at zero increase of surface pressure (origin of the abscissa in Figure 5) correspond to experiments in which MBP was injected beneath the lipid monolayer set at the "cut-off" pressure prior to film transfer; no penetration is possible at this pressure (Fidelio et al., 1982, 1986a,b) (see also Figure 2). The activities measured after the surface pressure is changed to 15

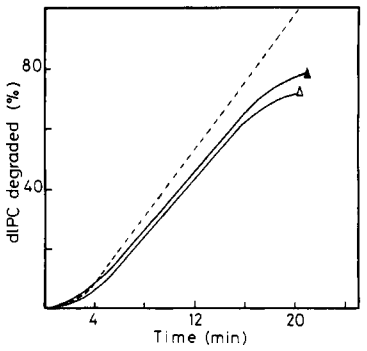


FIGURE 6: Effect of MBP on the extent of dlPC degradation. Percentage of dlPC degraded as a function of time after injecting at time zero 0.025 μg/mL (final concentration in the subphase) of $PLA_2$ under a monolayer of dlPC:MBP (initial molar ratio, 99:1) (△) at 10 $mN \cdot m^{-1}$ or dlPC:MBP (initial molar ratio, 98:2) (▲) at 12 $mN \cdot m^{-1}$. The dashed line represents the activity against a film of pure dlPC. The reproducibility of the determinations is as stated for Figure 2.

$mN \cdot m^{-1}$ in monolayers containing MBP (see above) are lower than those obtained in films handled in the same manner but in the absence of MBP (the value taken as 100%). This indicates that the $PLA_2$ activity is modulated by MBP at various levels. At high surface pressures, MBP is extruded from the hydrocarbon chain region but remains adsorbed to the polar head groups (Fidelio et al., 1984, 1986b). These results also emphasize the very high sensitivity of $PLA_2$ to detect changes of the lipid–protein organization of interfaces containing Sulf and MBP.

Experiments with films of dlPC–GalCer (Figure 5b) show a progressive inhibition of the enzyme as the penetration of MBP is increased. With these films set initially at the "cut-off" surface pressure for MBP penetration (zero increase of surface pressure), the enzyme activity subsequently measured at 15 $mN \cdot m^{-1}$ is within 90–100% of the values found for control films of pure dlPC. This activity is even higher than that found for a film of pure dlPC set at the cut-off pressure for MBP penetration (the curve for dlPC in Figure 5b). This suggests that, above the cut-off pressure, MBP adsorbs even more favorably to films of pure dlPC than to those containing GalCer (Figure 6b).

(*c*) *Effect of MBP on the Extent of dlPC Degradation by $PLA_2$.* We also performed experiments under continuously variable surface composition during the course of the enzyme action as recently described (Bianco et al., 1990, 1991). In this case, the *same* mixture of dlPC–MBP was spread over the reaction compartment *and* the reservoir. With this technique we can investigate the extent to which the dlPC available can be hydrolyzed by the enzyme when the relative proportion of dlPC is continuously decreasing in the film by the enzymatic action. Obviously, the kinetics will not be zero-order under these conditions (Bianco et al., 1990, 1991). Figure 6 shows that at the molar ratios (dlPC to MBP of 99:1 or 98:2) in which the dlPC and MBP are homogeneously mixed (see section 1), duplicating the proportion of MBP does not affect the enzyme activity. The enzyme action permits the degradation of about 80% of the dlPC. At this point, the mole fraction of MBP has increased in the film to 0.24 (from the initial values of 0.01 or 0.02), and about 80% of the interfacial area is occupied by MBP (Fidelio et al., 1984). These results show that the enzyme remains associated with the interface containing MBP for a large number of catalytic cycles [cf. Jain and Berg (1989)] as previously reported for films without MPB (Bianco et al., 1990, 1991). When the proportion of MBP is higher the films become biphasic and the interpretation of the results is complicated. This is because, due to the random distribution of the phase-separated domains at the interface, it can not be controlled in a simple manner which portion of the inhomogeneous film is automatically transferred by the surface barostat onto the reaction compartment.

## *Conclusions*

The modulation of the activity of $PLA_2$ depends on the relative amounts of MBP in the interface, on the type and proportions of GSLs, and on the lateral surface pressure as well as the sequence in which the components interact or are integrated into the interface. The modulation is different if MBP is an initial constitutive part of the lipid–protein monolayer or if it interacts with the lipid film from the subphase. It was described before that the effects of GSLs on the enzyme activity were not due to a direct or classical inhibition or activation of the lipids on the enzyme molecule (Bianco et al., 1990, 1991). Likewise, the marked dependence of the modulation by MBP on the surface conditions, the experimental setup itself (elimination of the subphase protein by film transfer), and the fact that the lag times for the reaction are not modified indicate that the effects are not due to a direct interaction of MBP with $PLA_2$ in the subphase solution. However, at the present stage, our results can not rule out that direct MBP–$PLA_2$ interactions may occur at the interface itself where they become markedly modulated by the surface conditions. On the other hand, it is important to consider that MBP is not a single entity but exists in several isomeric forms with different charge and size heterogeneity that interact differently with lipids (Wood & Moscarello, 1989). The different isomers have different effects on a brain phosphatidylinositol-specific phospholipase C from bovine brain, depending on their net cationic charge (Tompkins & Moscarello, 1991).

Our results constitute the first direct study, obtained under surface conditions controlled continuously during the reaction, of the molecular factors and interactions that regulate phospholipid degradation in interfaces containing GSLs and MBP. Several physicochemical parameters that modulate the phospholipase activity have been identified (Bianco et al., 1989, 1990, 1991). Very subtle variations of the lipid–protein composition and organization of the membrane interface result in dramatic alteration in its capability for being attacked by an exogenous hydrolytic enzyme. Characterization of the interplay of the various factors involved in these effects using membranes of different composition is essential for understanding the molecular events able to trigger, amplify, or impede a cascade of membrane phenomena that lead to the alteration and degradation of membrane components.

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