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Regulation of Phospholipase A_2 Activity by the Lipid–Water Interface: a Monolayer Approach†

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ABSTRACT: Interfacial regulation of phospholipase A_2 activity on lecithin monolayers was investigated by using radioactively labeled enzyme. Labeling of the protein with ^{125}I did not produce a change of the enzyme and protein properties as compared to the ^3H fully amidinated phospholipase A_2. The induction time observed during pre-steady-state kinetics reflects the rate-limiting step of the penetration of the enzyme in the interface. This penetration is reversible. However, in the surface pressure range where the enzyme is able to hydrolyze the lecithin films, the desorption of the protein from the film is slow as compared to the adsorption. Below a surface pressure of 10 dyn/cm nonspecific adsorption occurs. Using lecithins with fatty acids of different chain lengths, we have shown that the kinetics of the penetration process is governed by the packing density of the substrate molecules independent of the surface pressure. However, the steady-state surface concentration of the enzyme increases with the fatty acyl chain length of the lecithin, indicating that hydrophobic interaction occurs between phospholipase A_2 and the lipid molecules at the interface. From the lecithins used pancreatic phospholipase A_2 preferentially splits substrate molecules with nine carbon atoms in the acyl chain.

The marked increase in activity of lipolytic enzymes, such as phospholipases, induced by membranelike lipid structures indicates a very attractive model system to investigate specific regulations of enzymatic activity by various organized lipid–water interfaces.

For pancreatic phospholipase A_2 (EC 3.1.1.4), it has been proposed that a protein surface region is present, which recognizes and interacts with the lipid phase, the so-called interface recognition site (IRS)[1] (Verger et al., 1973; Pieterson et al., 1974a; van Dam-Mieras et al., 1975). The zymogen, which differs only by an additional seven amino acid residue on the N-terminal part, is devoid of this IRS and does not bind to lipid–water interfaces.

For the study of lipid–protein interactions, the monolayer technique is valuable because it is possible to regulate and measure the packing properties of a pure (phospho)lipid film without addition of surfactants. It has been shown that the interfacial properties such as surface pressure and charge of the lipid greatly affect the enzymatic catalysis at the lipid–water interface (Dawson, 1966; Verger et al., 1976). Verger & de Haas (1973) described a monolayer technique where surface pressure is kept constant during hydrolysis. By this technique, it is possible to measure pre-steady-state kinetics. After injection of the enzyme, the velocity increases with time and approaches an asymptotic limit. This kinetics can be described by two parameters: the slope of the asymptote V (velocity) and the lag time τ. For a review, see Verger & de Haas (1976). Verger et al. (1973) proposed a general model for the action of a soluble enzyme at an interface (Figure 1).

These authors proposed that the lag time reflects the first step of penetration of the enzyme in the lipid–water interface

$$E \underset{K_d}{\overset{K_p}{\rightleftharpoons}} E^*$$

Above a given surface pressure, the lag time increases abruptly, and a critical surface pressure exists where the enzyme can no longer penetrate the interface ($\tau \rightarrow \infty$).

The aim of this work was to investigate the validity of the model by using radioactively labeled porcine phospholipase A_2 and to determine which parameters of the lipid–water interface regulate the enzymatic activity.

Materials and Methods

Enzymes. Porcine phospholipase A_2 and its zymogen were prepared as described previously (Nieuwenhuizen et al., 1974).

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[1] Abbreviations used: AMPA, ε-amidinated phospholipase A_2; AMPREC, ε-amidinated prophospholipase A_2; PA_2, phospholipase A_2; IRS, interface recognition site; L-di-C8-PC, L-di-C9-PC, L-di-C10-PC, and L-di-C12-PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine with two identical acyl chains with 8, 9, 10, and 12 atoms of carbon, respectively; D-di-C10-PC, 2,3-didecanoyl-*sn*-glycero-1-phosphocholine; C6(1)-C12(3)PC, 1-hexanoyl-3-dodecanoyl-*sn*-glycero-2-phosphocholine; C12(1)-C6(3)PC, 1-dodecanoyl-3-hexanoyl-*sn*-glycero-2-phosphocholine.

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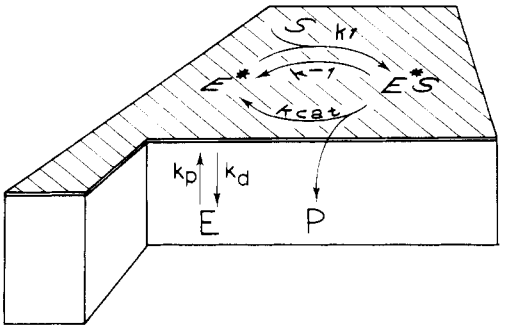

FIGURE 1: Proposed model for the action of a soluble enzyme at an interface [from Verger et al. (1973)]. K_m^* is defined as $(k_{-1} + k_{cat})/k_1$.

The exact enzyme concentration in solution was determined spectrophotometrically at a wavelength of 280 nm. Values of 13.0 and 12.3 for $E_{1\ cm}^{1\%}$ were used for phospholipase A_2 and the zymogen, respectively. ϵ-Amidinated prophospholipase A_2 (AMPREC) and phospholipase A_2 (AMPA) were prepared as described previously (Slotboom & de Haas, 1975). The [^{3}H]AMPREC was obtained by the same method on a 200-mg scale by starting from 0.3 g of cold acetonitrile mixed with 100 mCi of [^{3}H]acetonitrile from New England Nuclear. Reaction of the ϵ-amidinated zymogen with 2,4-dinitrofluorobenzene, followed by 6 N HCl hydrolysis for 18 h and amino acid analysis on the short column (Beckman Unichron amino acid analyzer), showed the quantitative conversion (>99.9%) of lysyl side chains into ϵ-acetimidolysine residues (Wofsy & Singer, 1963).

[^{3}H]AMPREC was purified on a DEAE-cellulose column equilibrated at pH 7.3 (0.005 M Tris) by using a NaCl gradient reaching 0.12 M. The specific radioactivity was 16.2 × 10^6 dpm/mg of enzyme (100 Ci/mol) which is close to the expected value for the number of lysine residues on the enzyme.

[^{3}H]AMPA was obtained by activation of [^{3}H]AMPREC with trypsin and purified on a CM-cellulose column at pH 6.0 (0.005 M acetate) by using a NaCl gradient reaching 0.4 M. [^{125}I]AMPA containing one atom of iodine per molecule of enzyme was prepared by adding an equimolar quantity of ^{125}I-labeled iodine (Radiochemical Centre, Amersham) to the enzyme (Slotboom et al., 1978b). The enzyme was then purified on a DEAE-cellulose column. The specific radioactivity was 6.5 × 10^7 dpm/mg of enzyme, which corresponds to 1.1 atoms of iodine per molecule of enzyme. Enzymatically inactive [^{3}H]AMPA was prepared by blocking the active site residue His-48 with 1-bromo-2-octanone as described by Volwerk et al. (1974) and Slotboom et al. (1978a).

Film Constituents. The short-chain 1,2-diacyl-*sn*-glycero-3-phosphocholines with two identical acyl chains (C8, C9, C10, and C12) were prepared as described by Cubero Robles & van den Berg (1969) while 2,3-didecanoyl-*sn*-glycero-1-phosphocholine was prepared by the method of Bonsen et al. (1972a). All lecithins were monitored for purity by thin-layer chromatography. 1-Dodecanoyl-3-hexanoyl-*sn*-glycero-2-phosphocholine and 1-hexanoyl-3-dodecanoyl-*sn*-glycero-2-phosphocholine were prepared as described by Slotboom et al. (1976).

Aqueous Subphase. Deionized water was distilled in an all-glass apparatus. Remaining tensioactive impurities were removed by sweeping and suctioning the surface before each assay. In all assays the aqueous subphase was composed of a 10 mM Tris–acetate buffer adjusted at a given pH in 0.1 M NaCl and 0.02 M $CaCl_2$.

Surface Barostat and Film Transfer. The surface barostat and the "zero-order trough" are identical with those described by Verger & de Haas (1973). The transfer trough and transfer experiment have been described previously (Rietsch et al., 1977).

Film Recovery and Film Counting. The film was recovered as described previously (Rietsch et al., 1977) directly in a counting vial by aspiration through a glass capillary. Radioactivity was counted in a Searle Isocap 300 scintillation system (Nuclear Chicago Division) by using 10 mL of a liquid scintillation cocktail (Packard Instagel).

Washing of the Trough. Because of adsorption of proteins on the Teflon walls of the trough, the washing procedure is essential after each experiment. The trough was washed with tap water and distilled water, then gently brushed in the presence of ethanol, and subsequently washed again with distilled water and bidistilled water.

In previous work (Verger et al., 1976) detergent was used instead of alcohol. Although extensive washing was done after the detergent treatment, traces of detergent remained, probably adsorbed on the Teflon. During the experiments, this amount of negatively charged detergent desorbed from the Teflon and adsorbed slowly in the lipid monolayer, changing its charge. This effect gave rise to a better and cooperative penetration of pancreatic phospholipase A_2 at high surface pressure on neutral lecithin films. The phenomenon was not observed on negatively charged films like phosphatidylglycerol or phosphatidylethanolamine. We checked that the cooperativity of enzyme adsorption was not due to the protein itself or fatty acid production. Upon removal of detergent during the washing procedure, this cooperative adsorption disappeared. This explains some of the quantitative discrepancies between the results described in this and the following papers and already published papers.

Results

Comparison of [^{3}H]AMPA and [^{125}I]AMPA Acting on Di-C10-lecithin Monolayers. Incorporation of radioactive markers in enzymes might change some of the kinetic properties of the native protein. Therefore, two differently labeled enzyme preparations were used, allowing a quantitative comparison of their kinetic behavior. Figure 2 represents the activity and the lag time profiles at pH 6.0 of [^{125}I]AMPA and [^{3}H]AMPA, respectively. The behaviors of these enzymes are quite similar and the minimal specific activities[2] are identical. The introduction of one iodine on tyrosine-69 of the enzyme (Slotboom et al., 1978b) slightly increases the penetration capacity of AMPA as shown by the lag time profile. Below 10 dyn/cm the [^{125}I]zymogen also binds to the film, but no activity could be detected. The behavior of the [H^3]zymogen is identical with that of the iodinated AMPREC (not shown in Figure 2B). Above 10 dyn/cm only the active enzyme is able to penetrate the lipid–water interface, and specific activity remains constant.

Relationship between Lag Time and Adsorption Process. Figure 3 shows the adsorption kinetics of [I^{125}]AMPA on L-di-C10-lecithin films at 12 dyn/cm at pH 6.0 and 8.0. At pH 6.0 within 2 min the surface radioactivity and the rate of hydrolysis are constant. At pH 8.0, however, the amount of enzyme in the film slowly increases with time, and only after about 25 min are a constant surface radioactivity and hydrolysis rate observed. During the penetration process, the specific activity of the enzyme remains constant.

Reversibility of the Adsorption. As shown in Figure 4, [^{125}I]AMPA injected under an L-di-C10–PC film at 10

[2] The minimal specific activity has been defined by Verger et al. (1976). It is calculated from the experimental velocity divided by the amount of protein at the interface.

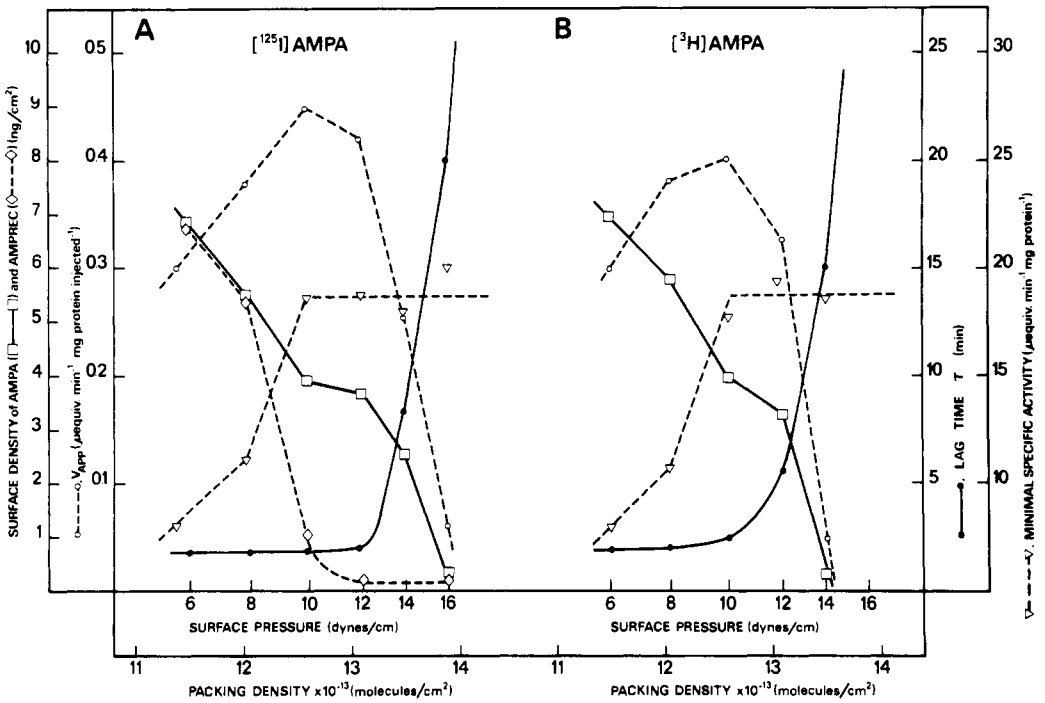

FIGURE 2: Influence of surface pressure, c.q. packing density on the activity, lag time, and surface density of [^{125}I]AMPA (A) and [^{3}H]AMPA (B) acting on L-di-C10-PC monolayer. Tris–acetate buffer (10 mM), pH 6.0; NaCl (0.1 M); $CaCl_2$ (0.02 M). Enzyme (15 μg) was injected. Volume of the reaction compartment was 210 mL; surface was 92 cm^2. The points on the specific activity curves were obtained by multiplying the ratio V_{app}/surface density by 1000/92.

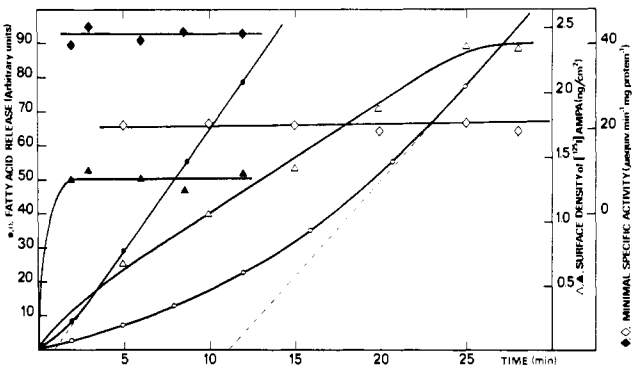

FIGURE 3: Adsorption kinetics of [^{125}I]AMPA on L-di-C10-PC monolayers at 10 dyn/cm; Tris–acetate buffer (10 mM) at pH 6.0 (▲) and pH 8.5 (△); NaCl (0.1 M); $CaCl_2$ (0.02 M). At given reaction times the film was collected and radioactivity was counted as described under Materials and Methods. The fatty acid release at pH 6.0 and 8.5 as a function of time is given by the curves (●) and (○), respectively. The dotted lines are the asymptotes to these curves. The velocity of the enzymatic reaction at any time of the kinetic run was obtained by determining the slope of the advancement curve. The points on the specific activity curves were calculated as given in the legend of Figure 2.

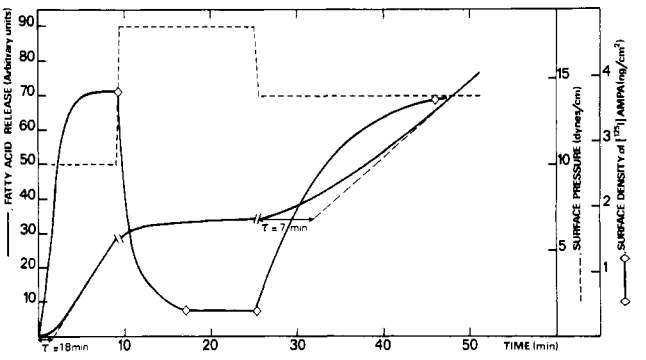

FIGURE 4: Reversibility of the penetration of [^{125}I]AMPA in L-di-C10-PC monolayer. Tris–acetate buffer (10 mM), pH 6.0; NaCl (0.1 M); $CaCl_2$ (0.02 M). [^{125}I]AMPA (12 μg) was injected.

dyn/cm and pH 6.0 reaches a steady state within 2 min. The film is then compressed up to a surface pressure of 18 dyn/cm. The enzymatic hydrolysis of the film stops abruptly (no production of fatty acids), and the surface radioactivity measurements show a rapid expulsion of the enzyme from the film into the bulk aqueous phase. Subsequent lowering of the surface pressure to 14 dyn/cm allows the enzyme to penetrate slowly in the film again, and the adsorption kinetics show the same lag time (7 min) as observed when the enzyme was injected directly under a film at 14 dyn/cm. For determination of the desorption rate of the enzyme from surface films into aqueous bulk phases devoid of enzyme, film transfer experiments were performed. The left part of Figure 5 shows the adsorption kinetics of [^{125}I]AMPA injected under a L-di-

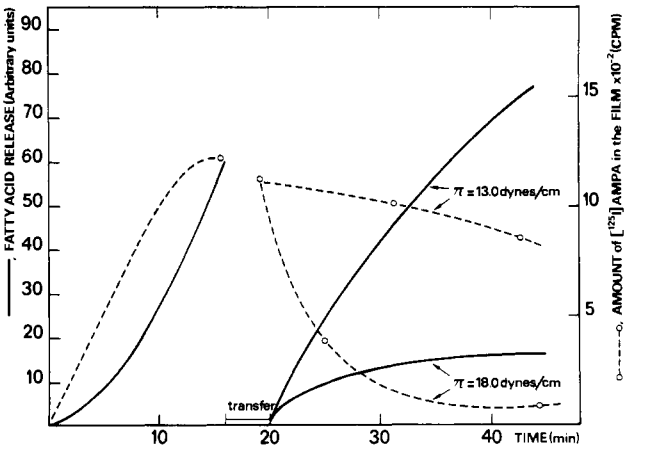

FIGURE 5: Desorption rate of [^{125}I]AMPA from L-di-C10-PC monolayers at 13 and 18 dyn/cm. Standard buffer, pH 6.0. The enzyme was first allowed to penetrate the film at 13 dyn/cm, and after reaching steady-state conditions, transfer of the film was performed at 13 dyn/cm to a bulk phase devoid of enzyme. [^{125}I]AMPA (6 μg) was injected.

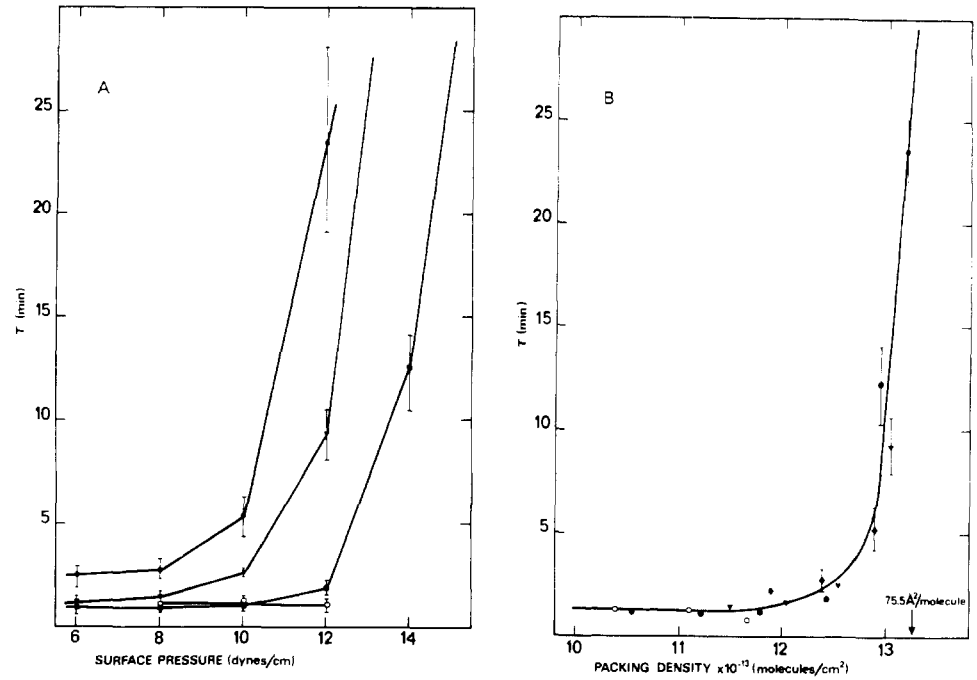

FIGURE 6: Influence of surface pressure (A) and lipid packing (B) of a lecithin film on the lag time. (○) Di-C8-PC; (●) di-C9-PC; (▼) di-C10-PC; (♦) di-C12-PC. Standard buffer, pH 8.0. [^{125}I]AMPA (8 μg) was injected.

C10-PC film at 13 dyn/cm. After about 15 min a steady state is reached, and the film is transferred at constant surface pressure to a fresh aqueous subphase devoid of enzyme. During this transfer no loss of protein from the film occurs and the initial velocity after transfer (at a time = 20 min) is identical with the velocity before transfer. However, during the next 20 min the fatty acid production slowly declines, and from surface radioactivity measurements it is clear that the enzyme slowly desorbs from the monolayer. As the rate of enzymatic hydrolysis decreases faster than expected from the surface radioactivity measurements, we must conclude that besides desorption a slow surface inactivation of the enzyme at the interface may also occur. At a surface pressure of 13 dyn/cm the "inactivation constant" can be calculated to be on the order of 10^{-2} min^{-1}. A similar experiment in which, immediately after transfer, the film was compressed from 13 to 18 dyn/cm shows a considerable increase in the desorption rate of the enzyme, and the enzymatic hydrolysis rapidly stops (Figure 5; π = 18.0 dyn/cm).

Influence of Lipid Packing on the Penetration Process. Figure 6A shows the lag time profile of [^{125}I]AMPA acting on lecithins of different chain lengths as a function of surface pressure. From these results it might be concluded that the penetration capacity of phospholipase A_2 increases when the fatty acid chain length is reduced. However, Zografi et al. (1971) showed that the lipid packing of short-chain lecithins of different chain length is different at the same surface pressure. Figure 6B gives a replot of the data of Figure 6A as function of the packing density of the molecules of phospholipids. Then all the points are falling on the same curve. The surface density where porcine phospholipase A_2 can no longer penetrate a lecithin film corresponds to 75.5 Å^2/molecule of phospholipid.

Effect of the Lecithin Chain Length on the Affinity and Specific Activity of AMPA. Figure 7 shows the surface density of AMPA during steady-state hydrolysis of di-C8-, di-C9-, di-C10-, and di-C12-lecithin monolayers as a function of lipid packing. One can see that AMPA can no longer penetrate the various lecithin films when the packing density exceeds 13.5 × 10^{13} molecules/cm^2. This critical packing

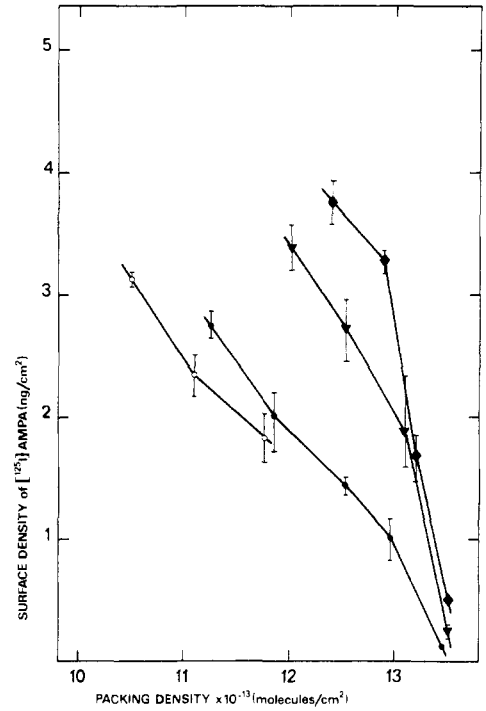

FIGURE 7: Influence of lipid packing of lecithin films on the surface density of [^{125}I]AMPA during steady-state hydrolysis. (○) Di-C8-PC; (●) di-C9-PC; (▼) di-C10-PC; (♦) di-C12-PC. Same conditions as given in Figure 6.

density corresponds with the sharp increase in the lag time profile of Figure 6B. From Figure 7, it is also clear that at constant packing density, the amount of enzyme in the substrate film increases with an increase of the fatty acid chain length of the lecithin. This indicates hydrophobic interaction between the enzyme and the lipid interface [cf. Soares de Araujo et al. (1979)]. The minimal specific activity of [^{125}I]AMPA acting on monolayers of lecithins with different chain lengths shows an optimum for dinanoyllecithin (Figure 8). Independent of the chain length, this minimal specific activity increases from 6 to 10 dyn/cm and remains constant

Table I: Activity of [^{125}I]AMPA on Stereoisomeric Lecithins[a]

substrate	surface pressure (dyn/cm) 10.0 % protein in the film	10.0 sp act. [(μequiv/min)/mg]	12.0 % protein in the film	12.0 sp act. [(μequiv/min)/mg]	14.0 % protein in the film	14.0 sp act. [(μequiv/min)/mg]
L-di-C10-PC	2.3	19.4	1.75	23.8	1.4	17.1
D-di-C10-PC			0.95	0		
C6(1)-C12(3)PC	7.1	0.24	4.3	0.43	3.1	0.67
C12(1)-C6(3)PC	5.7	0.1	2.6	0.25	1.6	0.26

[a] Tris-acetate buffer (10 mM), pH 8.0; NaCl (0.1 M); and $CaCl_2$ (20 mM).

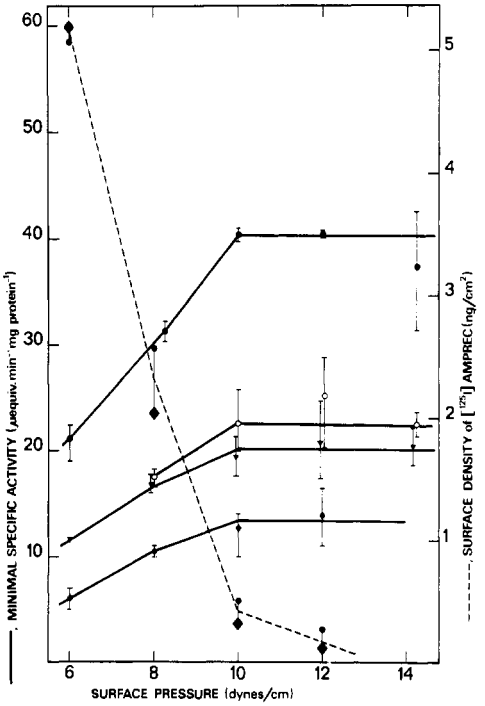

FIGURE 8: Influence of surface pressure on the specific activity of [^{125}I]AMPA acting on lecithins with different acyl chain length. (○) Di-C8-PC; (●) di-C9-PC; (▼) di-C10-PC; (♦) di-C12-PC. Same conditions as given in Figure 6. The dotted line represents the amount of [^{125}I]AMPREC which aspecifically adsorbs to di-C9- and di-C12-PC films.

at higher surface pressure. In Figure 8, the dotted line represents the surface density profile of the [^{125}I]AMPREC both on di-C9- and on di-C12-lecithin monolayers. Below 10 dyn/cm, the zymogen adsorbed to the film. However, no activity was detectable. Most probably, at these low surface pressures of the film an aspecific protein adsorption takes place, and therefore the specific activities of the active enzyme below 10 dyn/cm should be considered as apparent and minimal values.

Stereospecificity of the Penetration Process or Fatty Acid Effects. Table I gives the specific activities and surface density at steady-state conditions of [^{125}I]AMPA on L- and D-di-C10-lecithins and two stereoisomeric β-lecithins. As expected, monolayers of D-lecithins are not hydrolyzed by the enzyme, while β-lecithins are poor substrates (Bonsen et al., 1972b). More surprising are the differences found in the equilibrium surface concentration between the stereoisomers. A stereospecificity of the penetration process which could explain these results was ruled out by the fact that no difference in equilibrium surface concentration was observed when octanone-blocked enzyme was used or when Ca^{2+} ions were replaced by Ba^{2+} ions. In these latter cases no hydrolysis occurs but

Table II: Influence of Fatty Acid Production on the Surface Density of Octanone-[^{3}H]AMPA[a]

octanone-[^{3}H]AMPA (μg injected) (100%)	AMPA (μg injected)	lecithin film	% protein in the film	FA[b] production by AMPA (arbitrary units)
45	10	L-di-C10	3.8	5
45	0	L-di-C10	1.1	0.2
45	10	D-di-C10	0.5	0
45	0	D-di-C10	0.5	0

[a] Tris-acetate buffer (10 mM), pH 6.0; NaCl (0.1 M); $CaCl_2$ (20 mM). [b] FA, fatty acid.

the penetration step is known to be unaffected (Pieterson et al., 1974a,b). Taking into account the fact that at every surface pressure used there seems to be more protein in the film when the specific activity is higher, one is tempted to relate the equilibrium surface concentration of the enzyme to the rate of fatty acid and/or lysolecithin production. A direct relation between the rate of fatty acid production and the equilibrium surface concentration of enzyme could be demonstrated by using the octanone-inhibited [^{3}H]AMPA in the presence and absence of a small amount of nonradioactive but enzymatically active AMPA. As shown in Table II, the protein(s) injected under a D-lecithin film do not hydrolyze the film, and only 0.5% of injected protein(s) is present in the monolayer. A similar experiment with a film of L-lecithin shows a much higher percentage of injected protein present at the surface, and this percentage increases further upon greater fatty acid production in the presence of some AMPA. Although the fatty acids produced are readily soluble in water, there is a constant production of fatty acid during hydrolysis which might induce a transitory negative charge of the neutral lecithin monolayer.

Discussion

In the model given in Figure 1, it is assumed that upon injection of a water-soluble lipolytic enzyme under a lipid monolayer, the enzyme will penetrate reversibly into the film and hydrolyze the substrate monolayer. Because of the unfavorable ratio of interfacial area to bulk volume in the monolayer technique as compared with bulk kinetics, one may expect that the penetrated enzyme represents only a small fraction of the total protein injected. For the estimation of this amount of protein present in the surface film and for the determination of the specific activity of the enzyme, radioactively labeled enzyme is required. Recently, Verger et al. (1976), using ^{125}I-labeled porcine phospholipase A_2, directly showed that indeed only a very small amount of enzyme acts in the monolayer. Although specific iodination is the most easy labeling reaction for phospholipase A_2, the introduction of the iodine in the enzyme could give rise to erroneous results.

We therefore compared the interfacial properties of [^{3}H]-amidinated phospholipase A_2 with its ^{125}I analogue. It has been shown by Slotboom et al. (Slotboom & de Haas, 1975; Slotboom et al., 1978a) that the kinetic properties of AMPA are quite similar to those of native phospholipase A_2. Furthermore, a quantitative knowledge of the interfacial properties of AMPA appeared necessary because the amidinated protein was used as a reference compound in studies on the action of N-terminally modified phospholipase A_2 on substrate monolayers (Pattus et al., 1979). As shown in Figure 2, both enzymes have the same specific activity on lecithins at pH 6.0. We observed that this identity remains up to pH 10. It has been shown that, in addition to the N-terminal sequence, tyrosine-69 is also part of the IRS which is involved in the penetration of phospholipase A_2 into the lipid phase (H. Meyer, personal communication). The introduction of one molecule of iodine on this tyrosine slightly increases the penetration capacity. This is in agreement with the better K_m found for [^{125}I]phospholipase A_2 acting on micelles of di-C8-lecithin (Slotboom et al., 1978b). The optimum in enzymic activity found at 10 dyn/cm is only apparent. The amount of enzyme present at the surface during steady-state hydrolysis continuously decreases with increasing surface pressure. The specific activity increases up to 10 dyn/cm and then remains constant (Figures 2 and 7).

Figure 3 clearly shows that a long lag time corresponds to slow adsorption and that the induction time of the pre steady state is due to the penetration of the enzyme in the monolayer and not to a conformational change of enzyme already present in the film. Therefore, the lag time profile gives a good quantitative determination of the penetration capacity of the enzyme in the lipid–water interface.

Recently, it has been shown that phospholipase A_2 binds reversibly to lipid micelles with a high affinity (Slotboom et al., 1978a; Soares de Araujo et al., 1979). In contrast to the latter reports it has been claimed previously by Dervichian et al. (1973) that phospholipase A_2 adsorbs irreversibly to monolayers. Rietsch et al. (1977) also found by transfer experiments that no loss of enzyme from the film occurs within hours. However, these latter authors carried out their experiments at low surface pressure (4 dyn/cm), conditions which are known to favor irreversible surface denaturation of proteins (Rietsch et al., 1977). From Figures 4 and 5 it is evident that the desorption rate of phospholipase A_2 from an L-di-C10-lecithin monolayer at 18 dyn/cm is very high. However, Figure 5 also shows that at lower surface pressure, e.g., 13 dyn/cm, the desorption rate constant k_d is small as compared to the penetration constant k_p. Moreover, this transfer experiment shows that there is some inactivation of phospholipase A_2 at the interface, as was reported previously also for pancreatic lipase (Rietsch et al., 1977). In the last few years most of the work on lipolytic enzymes acting on substrate monolayers has been expressed as a function of surface pressure. From Figure 6 it is evident, however, that it is the lipid packing which governs the kinetics of penetration of phospholipase A_2 into the lipid–water interface independently of surface energy. The polar head group of the phospholipid molecule seems to play an important role in the kinetic aspect of this initial step. The penetration of the enzyme is much faster in more negatively charged monolayers (Verger et al., 1976). The interaction of AMPA with lecithin monolayers stops at a lipid packing of 75.5 Å^2/molecule (Figure 6B). With phosphatidylglycerol monolayers, AMPA can penetrate the interface up to 52.6 Å^2/molecule (Pattus et al., unpublished experiments). In the last few years, phospholipases have been extensively used to investigate the structure of natural membranes and to evaluate the equivalent surface pressure of the external monolayer of biological membranes [see, for example, Roelofsen & Zwaal (1976)]. The interpretation of such studies must take into account the lipid packing and surface charge dependency of these enzymes. For the same lipid packing, the surface energy of the lecithin monolayer decreases when the fatty acid chain length increases, due to increasing hydrophobic interactions between the chains. This has an effect on the equilibrium concentration of the enzyme in the lipid interface (Figure 7). The fact that the protein surface density increases with the chain length of the lecithin monolayer indicates that hydrophobic interactions are also important in the penetration process.

The effects of lipid packing and of fatty acyl chain length shown in Figures 6 and 7 suggest the following hypothesis. The activation energy of the binding process which governs the kinetics of penetration of the enzyme in the lipid interface is a function of the polar head group packing density. The protein must cross this energy barrier formed by the phospholipid head groups (or the organized water molecules around them). Then the enzyme penetrates between the phospholipid molecules, and hydrophobic interactions between the fatty acyl chains and part of the protein occur.

On the contrary, the zymogen adsorption to the surface film is rather aspecific and independent of the chain length of the lecithin used (Figure 8). Despite the fact that the active site is present in the zymogen (Pieterson et al., 1974a), no activity was detectable. This means that the interface recognition site is responsible not only for the penetration of the enzyme in the lipid phase but also for inducing an optimal orientation of the active site toward the phospholipid monomer at the surface. Aspecific adsorption of proteins at the air–water interface is a well-known phenomenon in surface chemistry (Davies & Rideal, 1963). Due to this effect, it is not possible to measure the real specific activity of porcine phospholipase A_2 toward lecithin surface film below 10 dyn/cm. Porcine AMPA possesses an optimal activity toward monolayers of lecithins with nine atoms of carbon in the fatty acid chains (Figure 8). This optimum cannot be directly correlated to individual kinetic constants of the reaction. In the kinetic model of Figure 1, the dissociation constant K_m* of the equilibrium

$$E^* + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES^*$$

is unknown. Such an optimum is probably caused by changes in K_m* and k_{cat} upon variation in fatty acyl chain length. Recently, Slotboom et al. (1976), using stereoisomeric β-lecithins with an acyl chain of six atoms of carbon in position 1 (or in position 3) and an acyl chain with 12 atoms of carbon in position 3 (or in position 1), showed indirectly that k_{cat} increases with an increase in the chain length of the released fatty acid while K_m* also increases. Unfortunately, it is not possible to measure activity on di-C6-lecithin monolayers and have a good comparison.

The experiments shown in Tables I and II demonstrate the sensitivity of pancreatic phospholipase A_2 for the surface charge of the lipid–water interface. Although they are highly soluble in water, the short-chain fatty acids are continuously produced during the lipolytic reaction and have an influence on the amount of phospholipase A_2 bound to the interface. To explain the results given by Figure 7 it might be argued that the lower solubility of the longer chain fatty acids will decrease their desorption rate from the film which would tend to increase the transitory negative charge of the lecithin monolayer

and result in a more effective penetration of the enzyme. In order the demonstrate that this is not the case for fatty acids with 8–12 atoms of carbon and that it is really hydrophobic interaction which is involved in the interfacial concentration of enzyme during steady-state hydrolysis, we repeated the experiments on lecithins of different chain length with octanone-blocked [^{3}H]AMPA. Octanone–[^{3}H]AMPA no longer displays enzymatic activity (no fatty acid production), but its interaction with lipid–water interfaces is identical with that of AMPA (Slotboom et al., 1978a). Octanone–AMPA again showed a higher equilibrium concentration upon an increase of the fatty acyl chain length of the lecithin.

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