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Site-Specific ϵ-NH_2 Monoacylation of Pancreatic Phospholipase A_2. 2. Transformation of Soluble Phospholipase A_2 into a Highly Penetrating "Membrane-Bound" Form†

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ABSTRACT: Long-chain lecithins present in bilayer structures like vesicles or membranes are only very poor substrates for pancreatic phospholipases A_2. This is probably due to the fact that pancreatic phospholipases A_2 cannot penetrate into the densely packed bilayer structures. To improve the weak penetrating properties of pancreatic phospholipases A_2, we prepared and characterized a number of pancreatic phospholipase A_2 mutants that have various long acyl chains linked covalently to Lys^{116} in porcine and to Lys^{10} in bovine phospholipase A_2 [Van der Wiele, F. C., Atsma, W., Dijkman, R., Schreurs, A. M. M., Slotboom, A. J., & De Haas, G. H. (1988) *Biochemistry* (preceding paper in this issue)]. When monomolecular surface layers of L- and D-didecanoyllecithin were used, it was found that the introduction of caprinic, lauric, palmitic, and oleic acid at Lys^{116} in the porcine enzyme increases its penetrating power from 13 to about 17, 20, 32, and 22 dyn/cm, respectively, before long lag periods were obtained. Incorporation of a palmitoyl moiety at Lys^{10} in the bovine enzyme shifted the penetrating power from 11 to about 25 dyn/cm. Only the best penetrating mutant, viz., porcine phospholipase A_2 having a palmitoyl moiety at Lys^{116}, was able to cause complete leakage of 6-carboxyfluorescein entrapped in small unilamellar vesicles of egg lecithin under nonhydrolytic conditions. Similarly, only this latter palmitoylphospholipase A_2 completely hydrolyzed all lecithin in the outer monolayer of the human erythrocyte at a rate much faster than *Naja naja* phospholipase A_2, the most powerful penetrating snake venom enzyme presently known.

Long-chain zwitterionic diacylphospholipids such as lecithin that form densely packed bilayer structures in water have been known for a long time to be very poor substrates for pancreatic phospholipase A_2 (Van Deenen & De Haas, 1963; De Haas et al., 1968). Only after transformation of the bilayer packing into mixed-micellar systems with the aid of detergents like sodium deoxycholate do long-chain phosphatidylcholines become good substrates for pancreatic phospholipase A_2. After the first reports of Op den Kamp et al. (1974, 1975) it is now well accepted, however, that small structural irregularities in the bilayer packing, induced by lipid-phase transition, also render the lecithin substrate liable to phospholipase A_2 hy-

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drolysis (Menashe et al., 1986). In agreement with these observations are the results of Wilschut et al. (1976, 1978) and Jain and co-workers (1973a,b). They showed that other structural defects in the bilayer caused, for example, by the high curvature of small unilamellar vesicles or by incorporation of lysophospholipids or alcohols also give rise to enhanced breakdown of the bilayer substrate by pancreatic phospholipase. Although it cannot be denied that such structural irregularities in the substrate bilayer improve the interaction of the enzyme with the lipid–water interface, the kinetics of the hydrolysis reaction remain extremely complicated. Usually long induction times are observed and the growing percentage of hydrolysis product in the bilayer exerts a strong influence on the kinetics of binding and catalysis. Not only in bilayer form but also when present as monomolecular surface films at the air–water interface, the zwitterionic lecithin molecule is attacked much less eagerly by the pancreatic phospholipase A_2 as compared to anionic phospholipids. Verger et al. (1973) and Pattus et al. (1979a–c) demonstrated a very slow anchoring of the pancreatic enzyme into lecithin surface films, and lag times of several minutes are quite common. What is the reason that similar snake venom phospholipases A_2, displaying a high sequence homology and strongly resembling three-dimensional folding (Renetseder et al., 1985), are able to anchor lecithin bilayers and surface films often without any lag time? Upon inspection of the high-resolution X-ray structures of the pancreatic phospholipases A_2 (Dijkstra et al., 1981, 1983) and of the *Crotalus atrox* enzyme (Keith et al., 1981; Brunie et al., 1985), it is evident that the lipid-binding domain of the pancreatic phospholipase A_2 contains less hydrophobic side chains than the corresponding surface area in many venom enzymes. It is the purpose of this study to investigate whether a series of chemically engineered pancreatic phospholipases, containing one single fatty acyl chain covalently attached to the enzyme, display better anchoring properties to lecithin bilayers and surface films.

Materials and Methods

Most of the materials and methods used in this study have been described in the preceding paper (Van der Wiele et al., 1988).

1,2-Didecanoyl-*sn*-glycero-3-phosphocholine (L-di-C_{10}-lecithin) was prepared as described by Cubero Robles and Van den Berg (1969). 2,3-Didecanoyl-*sn*-glycero-1-phosphocholine (D-di-C_{10}-lecithin) was obtained by incubation of *rac*-1,2-didecanoylglycero-3-phosphocholine with phospholipase A_2. *Naja naja* phospholipase A_2 was purchased from Sigma (St. Louis, MO). Egg lecithin was obtained from Lipid Products (Redhill, England) and 6-carboxyfluorescein from Eastman Kodak (Rochester, NY). The latter product was a generous gift of Drs. F. Dousseau and J. Dufourcq (Talence, France) and was purified according to the procedure of Ralston et al. (1981). All other chemicals used were of the highest quality available.

Leakage of 6-Carboxyfluorescein Entrapped in Egg-Lecithin Vesicles by Acyl-AMPAs.[1] An egg-lecithin suspension (10 mM) in the presence of 200 mM 6-carboxyfluorescein adjusted to pH 7.4 was sonicated 3 times for 2 min at 0 °C with a MSE 150-W sonicator. To separate the trapped and the untrapped 6-carboxyfluorescein, the vesicles were filtered through a Sephadex G-25 column, equilibrated with 50 mM Tris, 0.1 M NaCl, and 1 mM EDTA at pH 7.4, and used immediately after elution. All fluorescence measurements were done with a Jobin et Yvon JY 3D spectrofluorometer (excitation and emission slit widths were 4 and 10 nm, respectively) in a volume of 1.3–2 mL under constant stirring at 25 °C. 6-Carboxyfluorescein was excited at 490 nm, and emission was monitored at 515 nm. Fluorescence is self-quenched at high concentrations of 6-carboxyfluorescein due to dye–dye interactions (Weinstein et al., 1977). Incubations were started by the addition of the enzymes and the fluorescence (F) was measured. The same volume of distilled H_2O was added to the blank and the fluorescence (F_0) measured. Maximal leakage was determined by addition of Triton X-100 (final concentration 8×10^{-4} M) to the vesicles and measurement of the fluorescence (F_{Triton}). The percentage of leakage of 6-carboxyfluorescein was calculated from the formula $[(F - F_0) \times 100]/(F_{Triton} - F_0)$.

Hydrolysis of Lecithin in Intact Human Erythrocytes. Fresh human erythrocytes were washed twice with 150 mM NaCl and once with a buffer containing 140 mM NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 10 mM Tris, pH 7.5. Cell suspensions (5%) in buffer were incubated at 37 °C under gentle shaking with the various (acylated) phosholipases A_2. At times indicated, 5-mL aliquots from these incubation mixtures were transferred into 1 mL of 100 mM EDTA in saline and the cells collected by centrifugation. After one more wash with saline, lipids were extracted from the cells (Rose & Oklander, 1965) and separated by two-dimensional thin-layer chromatography (Broekhuyse, 1969). Hydrolysis of lecithin was calculated from the amounts of phosphorus (Böttcher et al., 1961) recovered from the lecithin and lysolecithin fractions, respectively. Parallel incubations of cells in buffer with either porcine [ϵ-NH_2-Lys^{116}]AMPA or [Pal-Lys^{116}]AMPA (in the latter case the buffer was supplemented with 20 mM EDTA) did not cause any detectable hydrolysis of lecithin. Hemolysis, determined as described by Roelofsen et al. (1971), never exceeded 2%. Scanning electron micrographs of human erythrocytes were recorded after 90 min of incubation with porcine pancreatic phospholipase or the acylated mutant enzymes as described previously (Kuypers et al., 1984).

Monolayer Experiments. The kinetic experiments were performed with the "zero-order trough" (Verger & De Haas, 1973) containing two compartments, a reaction compartment and a reservoir compartment connected by a small surface channel. Enzyme was injected under the film in the reaction compartment only, whereas the substrate film covers both compartments. The reservoir compartment contains a mobile barrier that was used to correct for substrate molecules removed from the film in the reaction compartment by enzymatic hydrolysis, thereby keeping the surface pressure (π) constant. The latter is measured in the reservoir compartment with a Wilhelmy plate (perimeter 3.94 cm) attached to a Beckman RIIC Model LM 600 electromicrobalance, which in turn is connected to a microprocessor that controls the movement of the mobile barrier. In the magnetically stirred reaction compartment the temperature was controlled by using a circulating water bath at 25 °C. During the monolayer experiment, data (bar position) are stored every 10 s into the internal memory of the microprocessor. After the experiment, data can be stored on diskettes for further analysis (Donné-Op den Kelder et al., 1984). With this technique accelerating kinetics are observed under certain conditions and long induction times are present (cf. Figure 1). This is caused by a slow reversible penetration of the enzyme into the monolayer. These lag times, τ, which are dependent on enzyme species, chemical structure of the substrate, and surface pressure of

[1] The abbreviations used have been described in the preceding paper (Van der Wiele et al., 1988).

Table I: Comparison of Characteristic Parameters of "Native" and Acylated Porcine and Bovine AMPAs on Monomolecular Surface Layers of L- and D-Di-C_{10}-lecithins

enzyme	max V_{app}[a]	π (dyn/cm)	$\Delta\pi_{max}$[b] for π_i = 0 (dyn/cm)	π_c[c] (dyn/cm) measured by kinetics using L-di-C_{10}-PC[d]	π_c[c] (dyn/cm) measured by surface pressure increase using D-di-C_{10}-PC[e]
porcine					
PLA	1.58	12	7.8	14	12.1
AMPA	0.23	10	9.2	13	15
[ϵ-NH_2-Lys[116]]AMPA	0.25	9	10.1	13	14.8
[Ac-Lys[116]]AMPA	0.072	9.5	9	13	13
[Mal-Lys[116]]AMPA	0.093	10	9.5	13	13.3
[Cap-Lys[116]]AMPA	0.27	14	14.3	17	18.4
[Lau-Lys[116]]AMPA	0.19	16	14.2	20	20.1
[Pal-Lys[116]]AMPA	0.11	25.5	15.4	32	28
[O1-Lys[116]]AMPA	0.18	19	14.5	22	20
bovine					
PLA	0.88	9	8.5	11	13.4
AMPA	0.14	9	8.7	11	11.3
[Arg[6]]AMPA	0.17	10	8.1	15	15.7
[Arg[6],Pal-Lys[10]]AMPA	0.16	16.5	15.8	25	24.7

[a] Maximal velocity of the enzymatic hydrolysis of a L-di-C_{10}-lecithin monolayer measured at the surface pressure indicated. The velocity is expressed in mm min^{-1} (μg of protein)$^{-1}$ injected, using a reaction compartment with a volume of 230 mL and a surface of 937 cm^2. Because the amount of enzyme present in the monolayer is unknown, the velocity is an apparent value. [b] $\Delta\pi_{max}$ for π_i = 0 represents the maximal surface pressure increase after extrapolation to zero value of the initial surface pressure. Values were obtained upon injection of the enzyme under a D-di-C_{10}-lecithin monolayer (see Figure 2B). [c] π_c is defined as the critical surface pressure. These values have been determined by linear extrapolation to zero surface pressure increase by using D-di-C_{10}-lecithin monolayers (data column 5). Alternatively, these values were estimated from the τ–π plots (kinetics of hydrolysis of L-di-C_{10}-lecithin monolayers) at which the τ values steeply increased (viz., at 20 min) (data column 4) (cf. also parts B and A of Figure 2, respectively). [d] Standard deviations are ±0.5, except for [Pal-Lys[116]] and [O1-Lys[116]] porcine AMPAs and [Arg[6],Pal-Lys[10]] bovine AMPA for which a standard deviation of ±1.0 was found. [e] Standard deviation ±3%.

the monolayer, characterize the penetrating power of the protein. The inset in Figure 1 shows that the lag time (τ) is independent of enzyme concentration (Verger et al., 1973). The analysis of the kinetic data was performed according to the method described by Verger et al. (1973). A program written in BASIC for nonlinear regression analysis based on the method of Fletcher and Powell (1963) was used. The unweighted kinetic data were fitted to the equation $P = V_m t + V_m\tau(e^{-t/\tau} - 1)$, where P is the bar position (mm) at time t (min), V_m the enzymatic velocity under steady-state conditions, and τ the induction time of the process that reflects penetration of the enzyme into the monolayer. From the above equation V_m can be deduced to be the slope of the titration curve when time approaches infinity, i.e., the asymptotic value ($t >> \tau$). Extrapolation of this asymptote to the x axis gives the value for τ (cf. Figure 1). In the case of rapidly penetrating enzymes (short τ values), the lag time can be measured manually. Slowly penetrating enzymes require the use of the computer in order to obtain accurate τ values.

The aqueous subphase was composed of 10 mM Tris, 0.1 M NaCl, and 20 mM $CaCl_2$ at pH 8. Residual surface-active impurities were removed before each assay by sweeping and suction of the surface. An aliquot of the lecithin solution in chloroform was spread over the aqueous subphase with a microsyringe, and several minutes were allowed to elapse for solvent evaporation to take place before the final value of the surface pressure was set.

Measurements of Penetration of Acyl-AMPAs into the Lipid Monolayer. The surface pressure increase due to adsorption of acyl-AMPAs to D-di-C_{10}-lecithin monolayers was measured by using a cylindrical trough drilled in a Teflon block (surface area 31 cm^2, total volume 50 mL). The aqueous subphase (10 mM Tris, 0.1 M NaCl, 20 mM $CaCl_2$ at pH 8) was continuously agitated as described previously (Gargouri et al., 1985).

Results

Monolayer Experiments. Figure 2A shows the surface pressure dependence of the induction time (τ) and of the

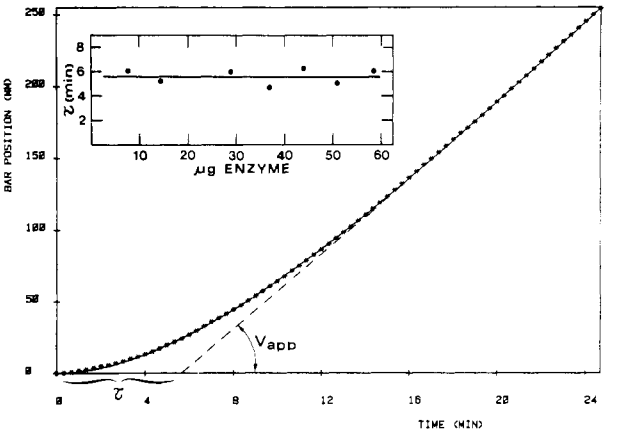

FIGURE 1: Kinetics of the hydrolysis of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine film at 10 dyn/cm upon injection of 30 μg of porcine AMPA. The curve represents the results of the computer fit, and the points on this curve represent the experimentally obtained data points. The slope of the asymptote (dotted line) to the curve represents the steady-state velocity (V_{app}). Extrapolation of this asymptote to the x axis gives the value for the induction time (τ). Experimental conditions: 10 mM Tris, 0.1 M NaCl, 20 mM $CaCl_2$, pH 8, 25 °C. Inset: Independence of lag timé (τ) from the amount of enzyme injected. Conditions: porcine AMPA (30 μ injected) at 10 dyn/cm.

apparent velocity (V_{app}) of porcine [Ac-Lys[116]]- and [Pal-Lys[116]]AMPAs acting on a L-di-C_{10}-lecithin monolayer. The τ–π curve of [Ac-Lys[116]]AMPA shows that this enzyme easily penetrates the monolayer at surface pressures (π) below about 13 dyn/cm before long lag periods are observed. This latter value represents the kinetically determined critical surface pressure π_c (cf. Table I, column 4). In sharp contrast, [Pal-Lys[116]]AMPA (Figure 2A; Table I, column 4) can penetrate much more densely packed monlayers up to a π_c value of about 32 dyn/cm before long lag times are observed. The critical surface pressures π_c of the various acylated porcine and bovine AMPAs are given in Table I (column 4). Figure 2A shows that the bell-shaped velocity–surface pressure curves have maximal values of the apparent velocities (max V_{app}) at

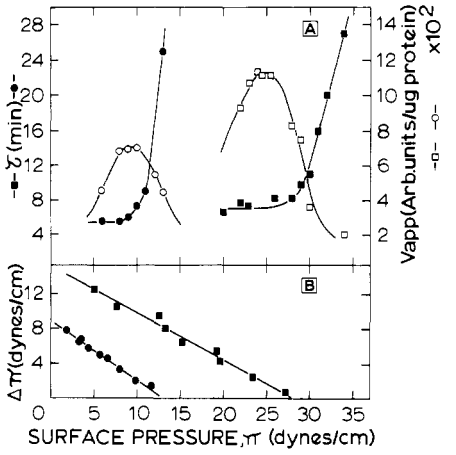

FIGURE 2: (A) Influence of the surface pressure (π) of a 1,2-didecanoyl-*sn*-glycero-3-phosphocholine monolayer on the induction time, τ (closed symbols), and on the velocity of the enzymatic hydrolysis, V_{app} (open symbols). Porcine [Ac-Lys^{116}]AMPA (○); porcine [Pal-Lys^{116}]AMPA (□). Conditions: 10 mM Tris, pH 8, 0.1 M NaCl, 20 mM $CaCl_2$, T = 25 °C. (B) Maximal increase in surface pressure ($\Delta\pi$) after protein injection with respect to the initial surface pressure (π) of a 2,3-didecanoyl-*sn*-glycero-1-phosphocholine monolayer. Porcine [Ac-Lys^{116}]AMPA (●), injection 100 μg; porcine [Pal-Lys^{116}]AMPA (■), injection 100 μg. Conditions: 10 mM Tris, 0.1 M NaCl, 20 mM $CaCl_2$, pH 8, T = 25 °C.

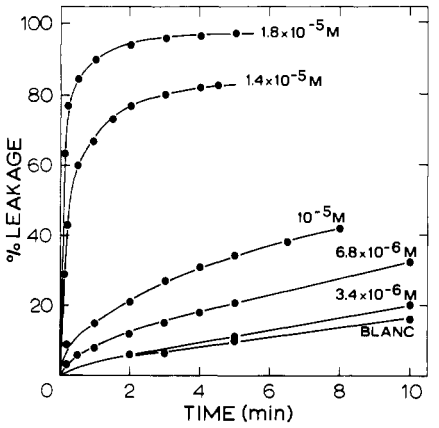

FIGURE 3: Percentage of leakage of 6-carboxyfluorescein entrapped in egg-lecithin vesicles as a function of the time upon incubation with increasing concentrations of porcine [Pal-Lys^{116}]AMPA. "Blanc" refers to incubation with addition of distilled H_2O or [ε-NH_2-Lys^{116}]AMPA (final concentration 2.9 × 10^{-5} M) instead of [Pal-Lys^{116}]AMPA. Conditions: egg lecithin, 0.75 × 10^{-5} M in 50 mM Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4 at T = 25 °C. For further details see Materials and Methods.

9.5 and 25.5 dyn/cm, respectively. Table I, first two columns, compiles the max V_{app} values and the surface pressures at which they are observed for the porcine and bovine PLAs, AMPAs, and their acylated analogues. We also determined the critical surface pressure values (π_c) of the acylated AMPAs by measuring the surface pressure increase of a D-di-C_{10}-lecithin monolayer upon injection of the enzyme. This method is much more rapid than the pre-steady-state technique but does not give information on velocity because no hydrolysis occurs. Figure 2B shows the linear dependencies of the surface pressure increase as a function of the initial surface pressure (π_i) for [Ac-Lys^{116}] and [Pal-Lys^{116}] porcine AMPAs, respectively. Upon extrapolation of the computer-fitted curves, π_c values of 13 and 28 dyn/cm, respectively, are found (cf. Table I, column 5). Extrapolation of the straight lines in Figure 2B to initial surface pressure zero yields $\Delta\pi_{max}$ values for [Ac-Lys^{116}]- and [Pal-Lys^{116}]AMPAs of 9 and 15.4 dyn/cm, respectively (cf. Table I, column 3). These values

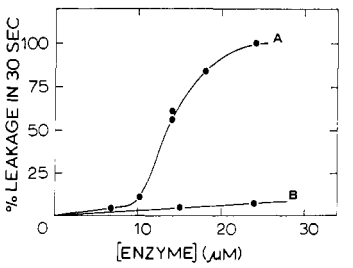

FIGURE 4: Percentage of leakage in 30 s of 6-carboxyfluorescein entrapped in egg-lecithin vesicles (egg-lecithin concentration 0.75 × 10^{-5} M) as a function of increasing concentrations of porcine [Pal-Lys^{116}]AMPA (curve A) and of bovine [Arg^6,Pal-Lys^{10}]AMPA, porcine [Lau-Lys^{116}]AMPA, and porcine [Pal-Lys^{116}]AMPREC (curve B). Conditions: see Figure 3 and also Materials and Methods.

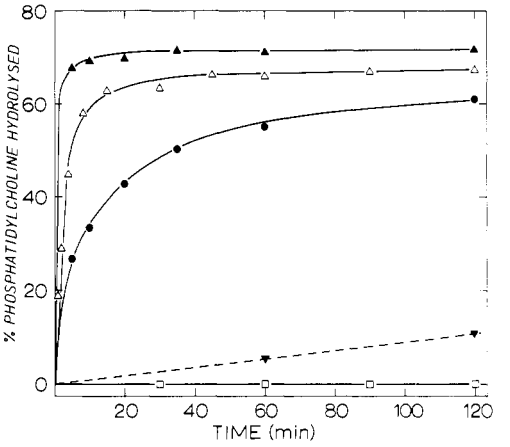

FIGURE 5: Percentage of hydrolysis of lecithin in intact human erythrocytes treated under nonlytic conditions with porcine [Pal-Lys^{116}]AMPA (▲, 90 μg; △, 3 μg), *Naja naja* phospholipase A_2 (●, 18 μg), porcine [Lau-Lys^{116}]AMPA and bovine [Arg^6,Pal-Lys^{10}]-AMPAs (▼, 120 and 600 μg, respectively), porcine [ε-NH_2-Lys^{116}]AMPA and porcine [Pal-Lys^{116}]AMPA (□, 50 and 90 μg, respectively). Conditions: see Materials and Methods. The indicated amounts of enzyme were added per 250 μL of packed cells. The incubation with porcine [Pal-Lys^{116}]AMPA (□) was done in the presence of 20 mM EDTA instead of Ca^{2+}.

reflect the surface-active properties of the enzyme and are given in Table I (column 3) for the various AMPAs and their acylated analogues.

Leakage of 6-Carboxyfluorescein Entrapped in Egg-Lecithin Vesicles by Acyl-AMPAs. Figure 3 shows the percentage of leakage as a function of time upon incubation of 6-carboxyfluorescein-containing vesicles with increasing concentrations of porcine [Pal-Lys^{116}]AMPA. At enzyme concentrations below 10^{-5} M there is a slow and limited release of the fluorescent dye. At enzyme concentrations above 10^{-5} M the leakage is much more rapid and almost quantitative. This is more clearly seen in Figure 4, showing the percentage of leakage of the dye in 30 s as a function of enzyme concentration. Curve A (Figure 4) represents the leakage of the dye mediated by increasing concentrations of porcine [Pal-Lys^{116}]AMPA. Between 10 and 20 μM [Pal-Lys^{116}]AMPA a steep increase in the leakage of the dye is observed. Similar incubations but with bovine [Arg^6,Pal-Lys^{10}]AMPA, porcine [Lau-Lys^{116}]AMPA, or porcine [Pal-Lys^{116}]AMPREC do not show this effet, and almost no leakage of the dye occurs in 30 s (Figure 4, curve B).

Hydrolysis of Lecithin in the Outer Monolayer of Intact Human Erythrocytes by Acyl-AMPAs. Upon prolonged incubations of healthy human red cells in serum with native porcine phospholipase A_2, [ε-NH_2-Lys^{116}]AMPA, or [Pal-Lys^{116}]AMPREC, no morphological changes occur. Short-

time incubations of the same cells with porcine [Pal-Lys^{116}]-AMPA, however, result in a dramatic morphological change of the erythrocytes into so-called "echinocytes".

Figure 5 shows the time-dependent breakdown of lecithin upon incubation of intact human erythrocytes with various phospholipases A_2. In the presence of native porcine pancreatic PLA, [ϵ-NH_2-Lys^{116}]AMPA, or [Pal-Lys^{116}]AMPREC, no hydrolysis of lecithin is observed even after 2 h of incubation (Figure 5, □). The same was found upon incubation with porcine [Pal-Lys^{116}]AMPA when the essential Ca^{2+} ions were replaced by EDTA. On the other hand, *Naja naja* PLA induces lecithin breakdown, albeit rather slowly (Figure 5, ●). After 2 h of incubation still no plateau value is reached. Although bovine [Arg^6,Pal-Lys^{10}]AMPA and porcine [Lau-Lys^{116}]AMPA have a higher penetrating power than their respective nonacylated AMPAs (Table I), they are able to hydrolyze only about 12% of the lecithin in the outer monolayer of the human red cell membrane in 2 h (Figure 5, ▼). However, a dramatic effect is seen upon incubation of the erythrocyte cell with porcine [Pal-Lys^{116}]AMPA. Figure 5 (▲) shows that this enzyme degrades the lecithin not only at a much higher rate but also much more efficiently than *Naja naja* PLA. The hydrolysis of lecithin reaches a plateau value within a few minutes. Even when the amount of porcine [Pal-Lys^{116}]AMPA is reduced 30-fold (Figure 5, △), the rate of hydrolysis is still considerably higher and the plateau is reached earlier as compared to the hydrolysis induced by *Naja naja* PLA.

Discussion

Since the first reports of Schmidt and Schlesinger (1979), the covalent attachment of lipid to protein is now a well-established posttranslational modification of polypeptides (Towler & Glaser, 1986; Etges et al., 1986; Chow et al., 1987; Sefton & Bush, 1987). The functions of protein-bound lipid moieties are as yet ill-defined, but they have been implicated in the mode of association of transforming gene products with the inner face of the plasma membrane. The covalent attachment of lipids to proteins by chemical means in order to improve certain enzyme properties was first reported by Drainas and Lawrence (1978). They showed a 100-fold activation of bee venom PLA_2 after incorporation of a single oleoyl residue. In 1985 Levashov et al. reported the incorporation of 1–2 stearoyl residues in trypsin and chymotrypsin. They suggested the possibility of translocation of these acylated proteolytic enzymes through the membranes of liposomes. A detailed study was reported recently by Babbit and Huang (1985). They covalently attached one single palmitic acid chain to Lys^{51} of α-bungarotoxin and immobilized and oriented the chemically homogeneous protein on the surface of a lipid vesicle by a process of spontaneous insertion via the acyl chain into preformed unilamellar vesicles.

With use of L-di-C_{10}-lecithin monolayers the kinetics of PLA hydrolysis are accelerated (Figure 1) and the curves obtained allow the determination of the apparent velocity as a function of surface pressure. From the bell-shaped velocity–surface pressure profiles (Figure 2) maximal apparent velocities can be derived (Table I). The introduction of a long acyl chain at Lys^{10} in bovine AMPA or at Lys^{116} in porcine AMPA does not significantly affect the maximal apparent velocities as compared with those of the corresponding nonacylated enzymes, except for [Pal-Lys^{116}]AMPA where it is reduced to about 50%. Introduction of a very short chain (Ac) or negatively charged short chain (Mal) diminishes the maximal apparent velocity much more, although not to such a low extent as observed for the specific activities in the egg-yolk assay or for the V_{max} values in the micellar L-di-C_8-lecithin assay (Van der Wiele et al., 1988). Furthermore, it is remarkable that the maximal apparent velocities of both porcine and bovine AMPAs are only 15% of the values of the respective native PLAs. Although a comparable drop in specific activity of these amidinated phospholipases A_2 is also observed in the egg-yolk assay, they possess about 75% of the V_{max} values of the corresponding native PLAs in the micellar L-di-C_8-lecithin assay (Van de Wiele et al., 1988). One has, however, to bear in mind that the reported maximal apparent velocities are based on the amount of enzyme injected in the subphase. These values are apparent because the amount of enzyme present in the monolayer is not known and they are measured at various surface pressures. This could give rise to different amounts of enzyme present in the monolayer. This phenomenon is most likely responsible for the above-mentioned differences in maximal apparent velocities.

In addition to the determination of the maximal apparent velocities, the kinetic monolayer experiments using L-di-C_{10}-lecithin simultaneously provide the critical surface pressure values (π_c) (Table I, column 4) at which the lag times τ steeply increase. These values, estimated from the τ–π profiles (Figure 2A) at a lag time τ of 20 min, correlate reasonably with the τ_c values obtained by measurement of the surface pressure increase under nonhydrolytic conditions using D-di-C_{10}-lecithin monolayers (Table I, column 5). It has to been mentioned that monolayers of stereoisomeric L- and D-lecithins are identical (Van Oort et al., 1985; Bonsen et al., 1972; Jain et al., 1986). Introduction of a short neutral or a somewhat longer but negatively charged maleyl group at Lys^{116} in porcine AMPA does not have any effect on its π_c value (Figure 2, Table I). Increasing the chain length of the acyl moiety at Lys^{116} in porcine AMPA to 10, 12, 16, or 18 carbon atoms dramatically increases the π_c values (Table I). The [Cap-Lys^{116}]- and [Lau-Lys^{116}]AMPAs can penetrate L-di-C_{10}-lecithin monolayers up to 17 and 20 dyn/cm, respectively, while [Pal-Lys^{116}]AMPA penetrates even up to 32 dyn/cm. From these data it can thus be concluded that the introduction of longer hydrophobic chains at Lys^{116}, located in the heart of the lipid-binding domain, renders the enzyme more penetrating. It is remarkable that [Ol-Lys^{116}]AMPA has a considerably lower π_c value (22 dyn/cm) than [Pal-Lys^{116}]AMPA (32 dyn/cm) despite the fact that the oleoyl moiety contains two more carbon atoms. One has, however, to bear in mind that the oleoyl chain, due to the presence of a cis double bond, has a more polar character than stearic acid. As a matter of fact, the hydrophobicity of the oleoyl chain is comparable to that of myristic acid. In this respect it is noteworthy that [Pal-Lys^{116}]- and [Ol-Lys^{116}]AMPAs have lower solubilities than native PLA, probably due to the higher tendency to aggregate, and cannot be purified by ion-exchange chromatography in aqueous solutions. No such problems are encountered for [Ac-Lys^{116}]-, [Mal-Lys^{116}]-, [Cap-Lys^{116}]-, and [Lau-Lys^{116}]AMPAs. The π_c value of [Arg^6,Pal-Lys^{10}] bovine AMPA is considerably lower than that of [Pal-Lys^{116}] porcine AMPA (Table I). Most likely this difference is due to the different position at which the palmitic chain is bound to the enzyme molecule. It does not seem probable that the hydrophobic acyl chain is fully exposed in the aqueous solution but that it will orient itself along a hydrophobic surface area at the enzyme surface. Obviously this orientation will be governed by the site of attachment to the enzyme molecule. Palmitic acid attached to Lys^{116}, located in the heart of the lipid-binding domain, will therefore reinforce the penetrating power much more than when it is linked to Lys^{10}, located

further away from the lipid-binding domain.

From the results of the monomolecular surface layer studies it is obvious that the acyl-AMPAs and in particular the porcine [Pal-Lys[116]]AMPA possess considerably improved penetrating capacities as compared to AMPA or native PLA. To determine whether these acylated enzymes can also penetrate densely packed bilayer structures, we studied the leakage of 6-carboxyfluorescein entrapped in small unilamellar vesicles of egg lecithin by fluorescence spectroscopy under conditions in which no hydrolysis occurs. From the results obtained it can be concluded that only the best penetrating mutant, viz., [Pal-Lys[116]] porcine AMPA, is able to cause complete leakage of the dye (Figures 3 and 4). Despite the improvement of their penetrating power, porcine [Lau-Lys[116]]AMPA, porcine [Pal-Lys[116]]AMPREC, and also bovine [Arg[6],Pal-Lys[10]]-AMPA have almost no effect (Figure 4). The remarkable steep increase of the leakage observed between 10 and 20 μM porcine [Pal-Lys[116]]AMPA could be indicative of aggregation of the protein, although no conclusive evidence is available yet.

It is well-known that native pancreatic PLA is unable to attack its substrates in the intact human erythrocyte (Roelofsen et al., 1971; Zwaal et al., 1975; Figure 5). Comparative studies with monomolecular (phospho)lipid films showed that this failure could be ascribed to the lateral surface pressure in the outer membrane leaflet of the erythrocyte, which is too high, probably 31–35 dyn/cm, for pig pancreatic PLA to penetrate this layer (Demel et al., 1975). *Naja naja* PLA, on the other hand, can attack the intact red cell and degrade the better part of its phosphatidylcholine, the major substrate, present in the outer membrane leaflet. This constitutes 70% of the total lecithin content in the red cell (Zwaal et al., 1975; Figure 5). However, its action ceases and the hydrolysis stops at a certain level before all of the lecithin in that layer has been degraded to completion (Zwaal et al., 1975). It has been suggested that this effect is a consequence of the production of the split products, lysolecithin and free fatty acids, which cause an increase in lateral surface pressure in the outer monolayer. This pressure may reach a certain level above which the enzyme can no longer penetrate (Roelofsen et al., 1980). In agreement with the above monolayer studies, it appears that [Pal-Lys[116]] porcine AMPA in contrast to [Lau-Lys[116]] porcine and [Pal-Lys[10]] bovine AMPAs is able to degrade the lecithin in the intact human red cell and, most interestingly, to do so at a much higher rate and with greater efficiency than *Naja naja* PLA (Figure 5). Even when the amount of [Pal-Lys[116]] porcine AMPA is reduced 30-fold, the rate of lecithin hydrolysis is still considerably faster than that induced by *Naja naja* PLA (Figure 5). The lecithin hydrolysis profiles shown in Figure 5 seem to suggest that [Pal-Lys[116]] porcine AMPA is firmly anchored in the outer membrane layer and is not therefore "squeezed" out of it so easily as is the *Naja naja* enzyme when the pressure in that layer increases due to the generation of the lecithin split products herein. The hydrolysis of lecithin in the outer monolayer by [Pal-Lys[116]] porcine AMPA transforms the erythrocytes into echinocytes. Incubation with native porcine PLA does not give hydrolysis, and consequently no echinocytes are formed.

From the present study it can thus be concluded that the covalent introduction of a long acyl chain in pancreatic PLA drastically changes its properties toward substrate present in densely packed bilayer structures. The effect is clearly dependent on the chain length, presence of double bonds, and position of linkage to the enzyme. Up until now the greatest effect is observed for palmitic acid linked to Lys[116], a position where it contributes optimally to the existing lipid-binding domain. Thus far we have assumed that in the presence of lipid–water interfaces the whole acyl chain remains in contact with the hydrophobic lipid-binding domain of the enzyme and thereby reinforces its lipid-anchoring properties. Alternatively, it has been proposed that the acyl moiety present in many proteins functions as a real anchor, i.e., that the long acyl chain inserts into the bilayer structure. The finding that [Pal-Lys[116]]AMPA can penetrate monolayers to higher pressure than [Ol-Lys[116]]AMPA is compatible with the "insertion" theory. The molecular area of palmitic acid is only 20 Å^2 and that of oleic acid 30 Å^2 (Davies & Rideal, 1961), which might prevent the latter's insertion into more densely packed monolayers. On the other hand, the differences in π_c values of [Cap-, [Lau-, and [Pal-Lys[116]]AMPAs are more in favor of the explanation that chain elongation renders the lipid-binding domain more hydrophobic by aligning itself along this domain. When insertion into the monolayer is assumed, it is not obvious why [Lau- and [Pal-Lys[116]]AMPAs, which have acyl chains that are two and six carbon atoms longer, respectively, than that of the substrate L-di-C_{10}-lecithin, lead to further improvement of the penetrating properties. At present our results do not allow discrimination between these two possibilities.

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