Activities and Interfacial Properties of *Rhizopus delemar* and Porcine Pancreatic Lipases after Treatment with Phospholipids†

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ABSTRACT: When sonicated with phospholipids, *Rhizopus delemar* and porcine pancreatic lipases acquire triacylglycerol hydrolyzing activity on an artificial lipoprotein substrate. Activities on tributyrylglycerol were unaltered by this treatment. Both native and phospholipid-treated *R. delemar* and porcine pancreatic lipases showed continuous increases in activity with increasing surface pressure on pure 1,2-didecanoyl-*rac*-glycerol. However, when a mixed 1,2-didecanoyl-*rac*-glycerol/1,2-didodecanoyl-*sn*-glycero-3-phosphocholine film was used as substrate, only 1,2-didecanoyl-*rac*-glycerol was hydrolyzed, and bell-shaped curves of activity vs. surface pressure were obtained. Phospholipid-treated lipases showed higher optimal surface pressures of action than those of the native enzymes. Critical surface pressures for penetration into a 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine film (not hydrolyzed) were raised remarkably upon the phospholipid treatment. It is proposed that phospholipid treatment would enhance the hydrophobic character of the lipid binding domain of the enzyme responsible for its penetration into organized lipid structures. These results indicate that both lipases were endowed with higher affinities for the mixed film after the phospholipid treatment and that phospholipid-treated lipases exhibit catalytic and interfacial properties comparable to those of milk lipoprotein lipase. The critical surface pressure of penetration into phospholipid film could thus be used as a criterion to distinguish further between lipase and lipoprotein lipase.

In the pancreatic juice of mammals, lipase activity is associated with a protein of about 50K daltons. It is well-known that homogenization of mammalian pancreatic tissue, as revealed after gel filtration, gives rise to a "fast" and/or a "slow" form of lipase (Sarda et al., 1964; Gelotte, 1964; Downey & Andrews, 1965; Fraser & Nicol, 1966; Schoor & Melius, 1969; Ramachandran, 1970; Kimura et al., 1972; Kovacs & Bezakova, 1972; Canioni et al., 1975; Rathelot et al., 1975). The fast lipase is characterized by its apparent high molecular weight. It is composed of aggregates of lipase molecules and lipids, including acidic phospholipids, fatty acids, and lysolecithins (Verger et al., 1969; Schoor & Melius, 1969). Fast lipase is highly active on olive oil emulsified in gum arabic. It can be converted into the slow form of the enzyme by the removal of lipids. Conversion to the slow form causes no change in the activity of the enzyme on olive oil and tributyrin emulsions. Since the original work on fast lipase (Sarda et al., 1964), no attention was paid to the possible kinetic differences between pure and lipid-associated pancreatic lipases.

Tamura et al. (1978) found that *Rhizopus delemar* lipase treated with pure phospholipid displayed appreciable triacylglycerol hydrolyzing activity on isolated human plasma lipoproteins. This observation was fully confirmed by Iwai et al. (1979), who reported that homogeneously purified *R. delemar* lipase exhibited only slight activity against synthetic lipid–protein association and that activity was increased 20–30 times after treatment with cardiolipin, lysophosphatidylcholine, or phosphatidylcholine. In contrast to this, phospholipid treatment had no effect on enzyme activity on emulsified olive oil. Subsequently, these authors found that binding of phospholipid to *R. delemar* lipase caused a decrease of the α-helix content of the enzyme and a shift of the isoelectric point to an acidic value (Iwai et al., 1980). The reversibility of the above phenomena was proved by depletion of the bound phospholipid from the phospholipid-treated lipase (Shimada et al., 1981).

Results obtained by Iwai et al. (1980) led us to the idea that lipase associated with phospholipid might be endowed with higher capacity to hydrolyze triacylglycerol in the form of natural or artificial lipoprotein. The present study was undertaken to better characterize activities and interfacial properties of phospholipid-treated pancreatic and microbial lipases.

Materials and Methods

Lipids. 1,2-Didodecanoyl-*sn*-glycero-3-phosphocholine ($diC_{12}PC$)[1] and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine ($diC_{16}PC$) were purchased from Fluka. Diphosphatidylglycerol (cardiolipin) was obtained from Sigma and was used without further purification. 1,2-Didecanoyl-*rac*-glycerol (dicaprin) was synthesized by Dr. J. Rietsch. Pure egg yolk lecithin giving a single spot upon thin-layer chromatography was a gift from Dr. P. Bougis.

Enzymes. Lipases from *R. delemar* and porcine pancreas were purified according to the methods of Iwai and Tsujisaka (1974) and of Verger et al. (1969), respectively. The starting material for *R. delemar* lipase was the commercial product of the enzyme (Tanabe Pharmaceutical Co., Japan). Porcine pancreatic lipase was checked free from colipase. Porcine

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[1] Abbreviations: *R.*, *Rhizopus*; LPL, lipoprotein lipase; PL, phospholipid; cardiolipin, diphosphatidylglycerol; dicaprin, 1,2-didecanoyl-*rac*-glycerol; $diC_{12}PC$, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine; $diC_{16}PC$, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; tributyrin, tributyrylglycerol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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pancreatic "fast" lipase prepared by the method of Sarda et al. (1964) was a gift from Germe-SA, France. Enzyme concentrations were estimated spectrophotometrically by using $E^{1\%}_{280nm}$ values of 11.2 for *R. delemar* (Iwai et al., 1980) and 13.3 for porcine pancreatic lipases (Verger et al., 1969).

Enzyme Assays. Both *R. delemar* and porcine pancreatic lipase activities were measured by titrimetry at pH 8.0, 25 °C, with a Radiometer pH-stat TTT 60, with 0.5 mL of tributyrin mechanically emulsified in 14.5 mL of 0.15 M NaCl solution containing 40 mM $CaCl_2$ (Erlanson & Borgström, 1970; Sémériva et al., 1971). Activities were measured by both the pH-stat technique and turbidimetry with an artificial lipoprotein substrate. This substrate was prepared by incubating 1 mL of a 5% bovine serum albumin solution in distilled water for 30 min at 30 °C with 40 μL of an emulsion obtained by mixing 8.8 g of olive oil with 100 mL of a 2% (w/v) gum arabic solution in water. A mixture containing 5 mL of the lipoprotein emulsion and 10 mL of 0.15 M NaCl solution was used for titrimetric assay with the pH-stat. The pH was maintained at 9.0 during the assay. For the turbidimeric assay (Iwai et al., 1979), a sample of 20–50 μL of lipase was added to a mixture of 0.2 mL of the lipoprotein emulsion and 0.8 mL of 0.2 M Tris buffer, pH 7.6, and then the turbidity decrease at 660 nm was followed. The initial absorbance at 660 nm was between 1.40 and 1.50.

Preparation of PL-Treated Lipase. The procedure was essentially based on the method of Iwai et al. (1980). Egg lecithin (1%, w/v) in 50 mM acetate buffer, pH 5.6, was sonicated for 5 min at 0 °C in a CIT-ALCATEL-PONS ultrasonicator 20/200S. The lecithin emulsion (0.1 mL) was added to 0.9 mL of *R. delemar* lipase solution (0.5–1.0 mg/mL in 50 mM acetate buffer, pH 5.6). The mixture was incubated for 5 h at 35 °C. After the incubation, an equal volume of an organic solvent mixture (isopropyl ether/1-butanol, 3/1 v/v) was added, and the whole mixture was shaken for 20 min at 30 °C at 60 oscillations/min to remove excess lecithin. Then, the solvent layer was discarded. This solvent treatment was repeated, and the water layer was kept in vacuo for 10 min to remove remaining solvent. Cardiolipin-treated porcine pancreatic lipase was prepared as follows: 6 mM cardiolipin in 10 mM Tris buffer, pH 8.0, was sonicated as described for egg lecithin. The emulsified cardiolipin (0.1 mL) was added to 0.9 mL of a pure pancreatic lipase, 0.5–1.0 mg/mL, in 10 mM Tris buffer, pH 8.0, free from colipase, and the mixture was incubated for 12 h at 0 °C. To remove excess cardiolipin, the solvent treatment was carried out twice as described above for the lecithin-treated *R. delemar* lipase.

Monomolecular Film Techniques. The surface barostat method (Verger & de Haas, 1976; Rietsch et al., 1977) was employed to determine the rate of hydrolysis of lipid monolayer at a constant surface pressure. We used a zero-order trough consisting of a reaction compartment (surface area 123 cm^2, total volume 210 mL) and a reservoir (surface area 500 cm^2). Before each experiment, the trough was cleaned with ethanol and rinsed with tap water and finally with distilled water. The aqueous subphase of the reaction compartment was thermostated at 25 ± 0.5 °C with an immersed glass coil and was agitated with two magnetic stirrers at 250 rpm. The surface of the aqueous phase was swept and aspirated to remove residual tensioactive impurities. Lipid in chloroform was then spread over the aqueous phase. Surface pressure was measured with a thin platinum plate (perimeter 3.94 cm) attached to a Beckman electromicrobalance LM 600. The platinum plate was washed with sulfochromic acid and rinsed with distilled water.

Measurements of hydrolysis of mixed-lipid monolayers were done as reported previously (Piéroni & Verger, 1979); a Teflon barrier was first placed transversely over the small canal of the trough to block surface communication between the reservoir and reaction compartment. Surface pressure was initially determined by placing the platinum plate in the reaction compartment where a mixed film (dicaprin/diC_{12}PC, 5/95 or 10/90 w/w) was spread at a desired surface pressure. Pure dicaprin was subsequently spread over the reservoir, and the surface pressure was adjusted to that in the reaction compartment by moving the Teflon barrier. Communication between the two films was then allowed by removing the barrier on the canal. Enzyme was injected into the reaction compartment, and the kinetics was recorded.

Measurements of Penetration of Lipase into Lipid Monolayer. Surface pressure increase due to the adsorption of lipase to lipid–water interface was measured in a cylindrical trough drilled in a Teflon block (surface area 31 cm^2, total volume 50 mL). The aqueous subphase was continuously agitated as described previously.

Labeling of R. delemar Lipase. R. delemar lipase was labeled with ^{125}I according to the lactoperoxidase method adapted from Thorell and Johanson (1971). The protein (1.5 mg) was dissolved in 0.5 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 120 nmol of sodium iodide. After addition of ^{125}I carrier free (1 mCi, Amersham), lactoperoxidase (Sigma) was added in a lipase to lactoperoxidase molar ratio of 1% in order to allow a low labeling level. Subsequently, 0.3 M hydrogen peroxide solution in the same phosphate buffer was added to the mixture (2 μL; 3 times at 2-min intervals). The whole mixture was exhaustively dialyzed against 6 L of 0.01 M acetate buffer, pH 5.6. The ^{125}I-labeled *R. delemar* lipase (sp radioactivity 28.8×10^{15} dpm/mol, corresponding to 1.6 atoms of iodine per lipase molecule) was then incubated with egg lecithin as described previously.

Film Recovery and Determination of Radioactivity. Fifteen minutes after injection of radioactive enzyme, corresponding to a steady state where the amount of adsorbed protein does not vary with time, surface film was collected by aspiration into a scintillation vial (Rietsch et al., 1977). As radioactive molecules dissolved in the subphase were unavoidably aspirated with the film constituents, results had to be corrected by counting radioactivity in the same volume of aspirated subphase. The difference between the two values, which actually expressed a certain excess of radioactivity existing at the surface, was attributed to the enzyme molecules bound to the film. Radioactivity was counted with a Packard Autogamma scintillation spectrometer Model 578.

RESULTS

Influence of PL Treatment on Activities of Lipases. (A) Hydrolysis of Artificial Lipoprotein. Although the results obtained by titrimetry and turbidimetry carried out with the artificial lipoprotein substrate cannot be quantitatively compared, both techniques show clearly that *R. delemar* and porcine pancreatic lipases acquired higher activities on this substrate when treated with phospholipids (Figure 1). However, triacylglycerol hydrolase activities measured with tributyrin as substrate were found to be unaltered by this treatment (data not shown). The treatment employed in this experiment thus increased activities of porcine pancreatic and *R. delemar* lipases on the lipoprotein substrate 2 and 16 times, respectively. The results on *R. delemar* lipase are consistent with those of Tamura et al. (1978), who used natural lipoprotein as substrate. Furthermore, we observed that higher activities on this substrate were accompanied with short lag

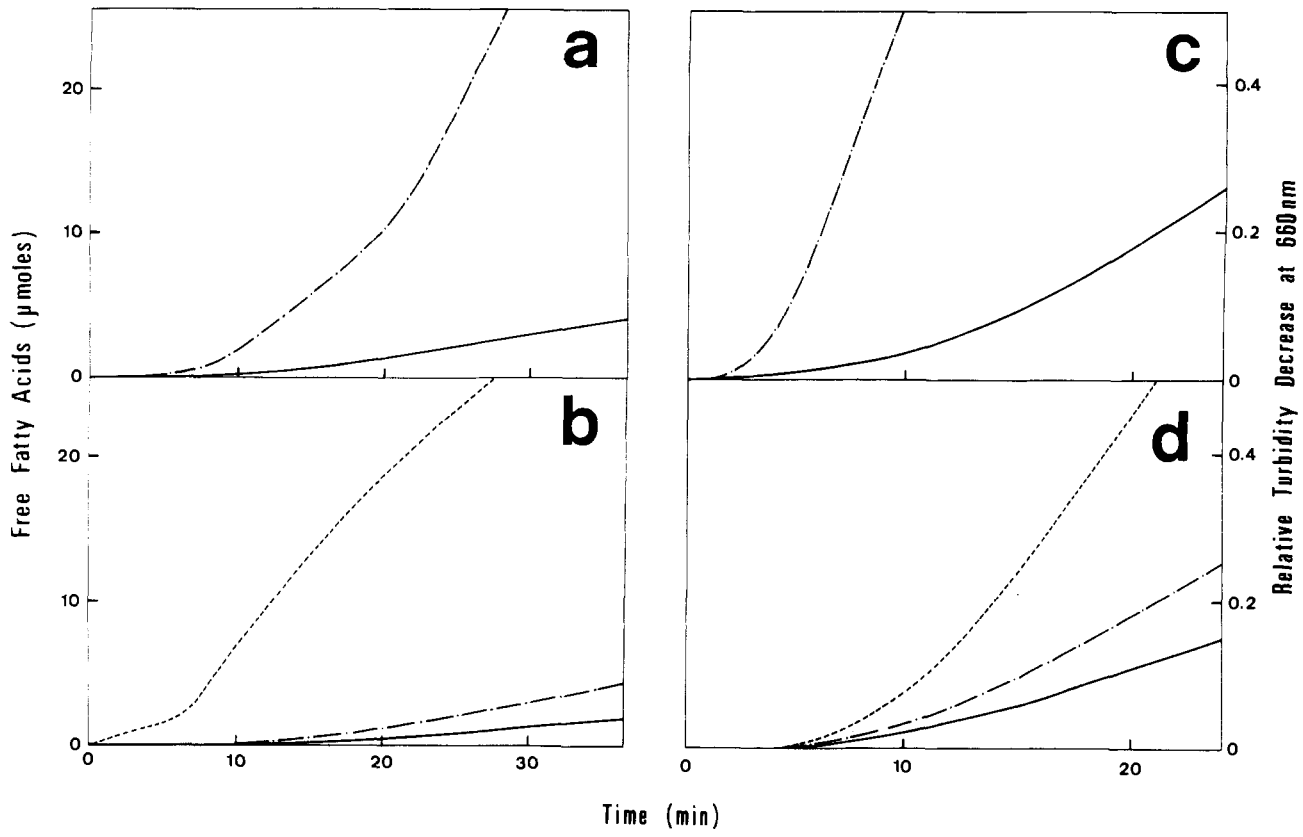

FIGURE 1: Lipase activity of PL-treated *R. delemar* and porcine pancreatic lipases. (Left panel) Measured by titrimetry at pH 9.0 with 3.6 μg of *R. delemar* (a) or 14.5 μg of pancreatic (b) lipase. (Right panel) Measured by turbidimetry at pH 7.6 with 0.35 μg of *R. delemar* (c) or 1.45 μg of pancreatic (d) lipase. (—) Native *R. delemar* or pancreatic lipase; (-·-) lecithin-treated *R. delemar* or cardiolipin-treated pancreatic lipase; (---) pancreatic fast lipase. Assay conditions are described under Materials and Methods.

time (data not shown). The presence of colipase reduced the lag time without affecting maximal activity of native or PL-treated pancreatic lipase.

(*B*) *Hydrolysis of Dicaprin Film.* Figure 2a,b indicates, the hydrolytic activities of *R. delemar* and pancreatic lipases measured on monomolecular films of pure dicaprin. Both lipases showed continuously increasing activities with surface pressure. Pancreatic lipase did not act significantly on the substrate film at surface pressures below 5 dyn/cm, while *R. delemar* lipase showed activity even at such low pressures, suggesting a higher resistance to surface denaturation (Rietsch et al., 1977). *R. delemar* lipase treated with egg lecithin as well as "fast" pancreatic lipase showed almost the same activities as those of the native enzymes. The activity of pancreatic lipase decreased slightly after cardiolipin treatment (Figure 2b).

(*C*) *Hydrolysis of Mixed Dicaprin/diC_{12}PC Film (5/95 w/w).* The absence of phospholipase activity of *R. delemar* and pancreatic lipases on pure diC_{12}PC allowed us to use the method of Piéroni and Verger (1979) to study the influence of diC_{12}PC on dicaprin hydrolysis. Figure 2c,d shows the hydrolytic activities of *R. delemar* and pancreatic lipases on these mixed films at different surface pressures. The treatment of *R. delemar* lipase with egg lecithin shifted the optimal surface pressure from 12 to 16 dyn/cm, as shown in Figure 2c. Native pancreatic lipase showed its maximal activity at 10 dyn/cm. When treated with cardiolipin, the activity profile slightly shifted to higher surface pressure, as shown in Figure 2d. Fast pancreatic lipase, a complex of pancreatic lipase with endogenous pancreatic lipids, showed maximal activity at 15 dyn/cm.

Influence of PL Treatment on Penetration of Lipases into diC_{16}PC. The increase in surface pressure of a lipid monolayer after protein injection could be interpreted by the penetration of a part of the protein into the lipid monolayer (MacRitchie, 1978). We ascertained that 680 μg of pure egg lecithin injected into the subphase of the cylindrical trough, which corresponded to 25 times the amount normally added with PL-treated lipases, did not cause any increase in surface pressure. Figure 3 shows plots of surface pressure increments against initial surface pressures. The critical surface pressure for penetration is the extrapolated value of the initial surface pressure above which no surface pressure increase is observed. The critical surface pressures of native *R. delemar* and pancreatic lipase were 9.5 and 19 dyn/cm, respectively. Incubation with phospholipids of these two lipases from microbial and mammalian origin caused a remarkable increase in the critical surface pressure of penetration to 50 and 44 dyn/cm, respectively.

Influence of PL Treatment on Binding of R. delemar Lipase to a Mixed Dicaprin/diC_{12}PC Film. The amounts of native and PL-treated ^{125}I-labeled *R. delemar* lipase adsorbed to a mixed dicaprin/diC_{12}PC film (5/95 w/w) were determined at variable surface pressures, simultaneously with their hydrolytic activities (Figure 4). The results show clearly that a greater amount of PL-treated ^{125}I-labeled enzyme is adsorbed to the interface as compared with the native ^{125}I-labeled enzyme. The activity–surface pressure curves of native and lecithin-treated ^{125}I-labeled lipases are comparable to their respective profiles obtained in the case of unlabeled lipases (see Figure 2c,d).

Discussion

There is presently large evidence that phospholipids are essential for the function of a number of membrane-bound enzymes. For most of them, the requirement for phospholipids

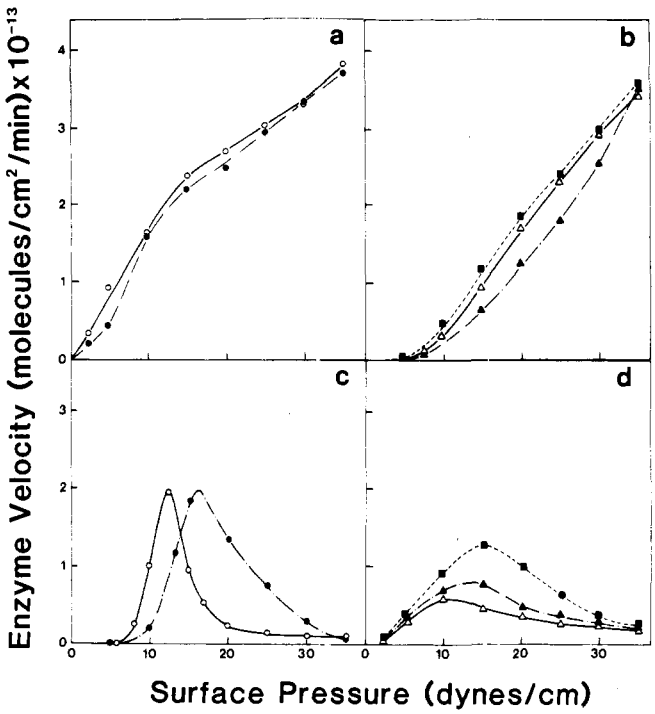

FIGURE 2: Influence of PL treatment on lipase activities. Rate of hydrolysis of pure dicaprin monolayers by *R. delemar* (a) and porcine pancreatic (b) lipases as a function of surface pressure: (○) native *R. delemar* lipase (0.025 μg); (●) lecithin-treated *R. delemar* lipase (0.025 μg); (△) native pancreatic lipase (0.29 μg); (▲) cardiolipin-treated pancreatic lipase (0.29 μg); (■) pancreatic fast lipase (0.29 μg). Rate of hydrolysis of dicaprin in mixed dicaprin/diC_{12}PC films by *R. delemar* (c) and porcine pancreatic (d) lipases as a function of surface pressure: (○) native *R. delemar* lipase (0.55 μg); (●) lecithin-treated *R. delemar* lipase (0.55 μg) [mixed dicaprin/diC_{12}PC (5/95 w/w) films]. (△) Native pancreatic lipase (0.4 μg); (▲) cardiolipin-treated pancreatic lipase (0.4 μg); (■) pancreatic fast lipase (1.0 μg) [mixed dicaprin/diC_{12}PC (10/90 w/w) films]. Assay conditions are described under Materials and Methods. Subphase was 0.1 M NaCl–1 mM EDTA, pH 8.0. For pancreatic lipase assays, the same buffer containing 21 mM $CaCl_2$ was used.

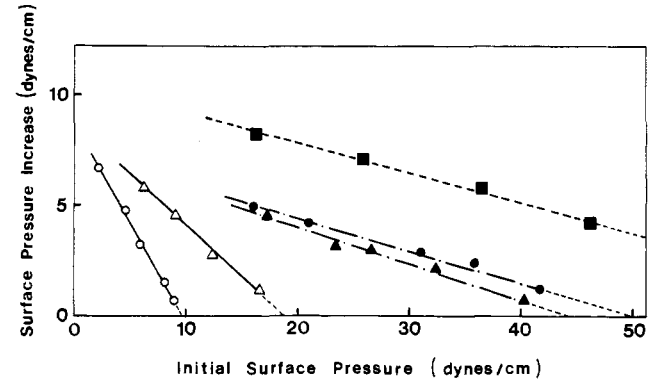

FIGURE 3: Interaction of *R. delemar* (0.74 nM) and pancreatic (2.2 nM) lipases with diC_{16}PC monolayer. Surface pressure increase as a function of initial surface pressure. The symbols are the same as in Figure 2. Subphase was 0.1 M NaCl–1 mM EDTA, pH 8.0. For pancreatic lipase assays, the same buffer containing 21 mM $CaCl_2$ was used.

seems to be nonspecific, but in some instances, as shown for mitochondrial D-β-hydroxybutyrate dehydrogenase, the inactive lipid-free enzyme can be reactivated specifically by lecithin (Gazzotti et al., 1975). L-Lactate dehydrogenase from *Escherichia coli* membranes is activated 3-fold by phosphatidylglycerol, cardiolipin, or a mixture of phospholipids with a concomitant 1.7-fold increase in its α-helical content (Kimura & Futai, 1978). It has been reported that liver esterase, after

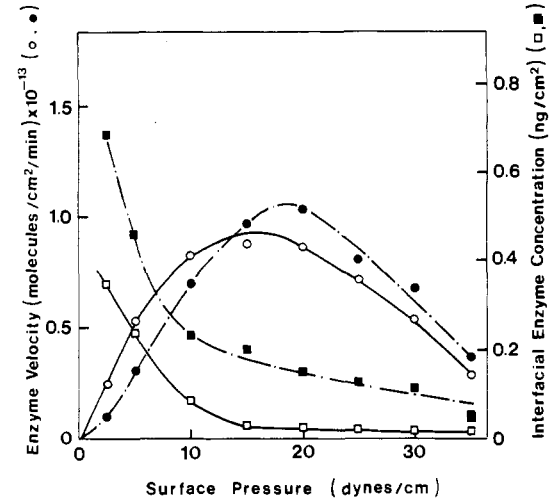

FIGURE 4: Enzymatic hydrolysis of dicaprin by ^{125}I-labeled *R. delemar* lipase (0.56 μg) in mixed monomolecular films of dicaprin/diC_{12}PC (5/95 w/w): (○) activity of ^{125}I-labeled *R. delemar* lipase; (●) activity of lecithin-treated ^{125}I-labeled *R. delemar* lipase; (□) surface excess of ^{125}I-labeled *R. delemar* lipase; (■) surface excess of lecithin-treated ^{125}I-labeled *R. delemar* lipase. Subphase was 0.1 M NaCl–1 mM EDTA, pH 7.4.

sonication in the presence of phospholipids, acquired the capacity of hydrolyzing emulsions of triacylglycerol (Okuda & Fiji, 1968). The binding of five phospholipid molecules to *R. delemar* lipase increases its activity on artificial lipoprotein substrate and induces a conformational change as revealed by CD spectroscopy (Iwai et al., 1980).

In this work, we have investigated the influence of phospholipid treatment upon the activities of pancreatic and *R. delemar* lipases. Emulsions of tributyrylglycerol or artificial lipoproteins as well as monomolecular films of dicaprin or mixed dicaprin/diC_{12}PC were used as substrates for both enzymes. By studying the increase of surface pressure (ΔΠ) as a function of the initial surface pressure of diC_{16}PC films (Π) resulting from a lipase–lipid interaction, we also evaluated the capacity of the enzymes to penetrate lipid structures. This study was aimed at correlating interfacial and catalytic properties of the PL-treated enzymes.

Native and PL-treated lipases (from pancreas and *R. delemar*) showed only small differences in their activity–surface pressure curves when acting on pure dicaprin monolayers (Figure 2a,b). This observation is consistent with the results that native and PL-treated lipases hydrolyze emulsified pure tributyrylglycerol (this work) or pure triolein (Iwai et al., 1979) at the same rate. These data show that pure porcine pancreatic lipase acquires higher activity on an artificial lipoprotein substrate after its treatment with cardiolipin (Figure 1). Comparable results have been previously reported for *R. delemar* lipase (Iwai et al., 1979). Lipid-associated lipase formed during pancreatic tissue homogenization (fast lipase) also shows higher activity against the artificial lipoprotein substrate as compared to the lipid-free enzyme (Figure 1b,d). Furthermore, from the kinetic curves shown in Figure 1, lag times are significantly reduced after PL treatment of lipases.

Bell-shaped velocity–surface pressure profiles were obtained with pancreatic and *R. delemar* lipases by using mixed dicaprin/diC_{12}PC films (Figure 2c,d). However, PL-treated lipases showed enzyme activity at higher surface pressures. These typical velocity–surface pressure profiles found presently with dicaprin or mixed dicaprin/diC_{12}PC monolayers by using pancreatic or *R. delemar* lipases are reminiscent to those found for lipoprotein lipase isolated from bovine milk acting on the same substrates (Vainio et al., 1983).

These results show that PL treatment of pancreatic and *R. delemar* lipases increases from 10–20 up to 40–50 dyn/cm the critical surface pressure above which enzyme injection under a phospholipid monomolecular film induces no surface pressure increase (Figure 3). Lipoprotein lipase from bovine milk was found to have a critical surface pressure into phospholipid monolayers around 50 dyn/cm (Vainio et al., 1983). Thus, PL-treated pancreatic and *R. delemar* lipases show catalytic and interfacial properties similar to those of milk lipoprotein lipase.

As recently shown by Remy et al. (1985), chemical modification of amino groups of chymotrypsin by aliphatic aldehydes increases maximal velocity of the hydrolysis of *N*-glutamyl-L-phenylalanine-4-nitroanilide to 164%. Authors have proposed to interprete this increase as resulting from microenvironmental effects on enzyme catalysis of the chemically introduced hydrophobic chains. For pancreatic phospholipase A_2, it has been found that specific acylation of lysine-116 with long-chain fatty acid enables the enzyme to penetrate densely packed monolayers and to attack biological membranes in contrast to the native enzyme (De Haas, 1985). For lipases, we propose that PL treatment enhances the hydrophobic character of the lipid binding domain responsible for penetration into organized lipid structures.

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Registry No. LPL, 9004-02-8; $diC_{12}PC$, 18194-25-7; $diC_{16}PC$, 63-89-8; dicaprin, 82950-64-9; lipase, 9001-62-1.

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