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Is Lateral Phase Separation Required for Fatty Acid To Stimulate Lipases in a Phosphatidylcholine Interface?[†]

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ABSTRACT: Lipase-catalyzed oxygen exchange between 13,16-*cis,cis*-docosadienoic acid and water in liquid-expanded monolayers with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine exhibits abrupt, lipid composition-dependent changes in extent and mechanism [e.g., Muderhwa, J. M. and Brockman, H. L. (1992) *J. Biol. Chem. 267*, 24184–24192]. The critical nature of this transition suggests possible lateral phase separation of the lipids. This has been addressed by substituting for either lipid species one which can exist in more condensed monolayer states. Analysis of phase transition surface pressures as a function of lipid composition shows that each set of fatty acid–phosphatidylcholine mixtures exhibits a finite range of miscibility in liquid-expanded monolayers. These results strongly suggest that 13,16-*cis,cis*-docosadienoic acid and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine are miscible under the conditions of the oxygen-exchange experiments. Furthermore, to address more directly the relation of lateral lipid phase separation to lipase regulation, oxygen exchange catalyzed by pancreatic carboxylester and triglyceride lipases was studied using mixed monolayers of [^{18}O]$_2$-docosadienoic acid and 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine. These lipids are miscible in the liquid-expanded state at all compositions. The lipid composition dependencies of both the extent and mechanism of lipase-catalyzed oxygen exchange were essentially identical to those obtained earlier. Thus, lateral lipid phase separation is not required for the critical transition in substrate accessibility to lipases. This finding supports a percolation-based model of lipase regulation within a single surface phase and suggests the "topo-temporal" regulation of lipid-mediated signaling in cells.

Free fatty acids are generated intra- and extracellularly by the hydrolysis of both neutral lipids, such as tri- and diacylglycerols and phospholipids. In addition to being building blocks for lipid remodeling and precursors for prostanoid synthesis, free fatty acids themselves are recognized as direct modulators of enzymatic activities. As examples, *cis*-unsaturated, but not *trans*-unsaturated or saturated, fatty acids are able to activate cellular protein kinases C, either directly or as potentiators of diacylglycerol stimulation (Nishizuka, 1992 and refs therein), and exogeneously-added fatty acids inhibit signaling in cytotoxic T lymphocytes (Anel *et al.*, 1993). It has been proposed that, in hepatocytes, the direct targets of fatty acid potentiation of diacylglycerol effects are phospholipase D and phosphatidate hydrolase (Siddiqui & Exton, 1992).

In more defined systems, fatty acids have been shown to be regulators of lipolytic enzymes. For example, fatty acids and other anionic additives promote the adsorption of phospholipase A$_2$ to zwitterionic phospholipid bilayers (reviewed in Ramirez and Jain (1991)). Effects of fatty acids on both interfacial enzyme adsorption and subsequent catalysis have been described for pancreatic carboxylester lipase (CEL)[1] acting at interfaces comprised of pure films of 13,16-*cis,cis*-docosadienoic acid (DA) (Muderhwa *et al.*, 1992) and mixed monolayers of DA and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Muderhwa & Brockman, 1992a). Fatty acid was the substrate in these studies, its catalytically-productive interaction with the enzyme having been monitored by lipase-catalyzed exchange of carboxyl oxygens with those of water (Muderhwa *et al.*, 1992). As the DA mole fraction was increased from 0.4 to 0.6 in liquid-expanded (LE) monolayers of POPC, the extent of DA oxygens exchanged by CEL added to the aqueous subphase increased abruptly (Muderhwa & Brockman, 1992a). This apparently critical transition in fatty acid availability to CEL can only in part be attributed to increased adsorption of the enzyme to the interface as DA content was increased (Tsujita *et al.*, 1987; Muderhwa & Brockman, 1990). Moreover, the phenomenon cannot be the result of product accumulation as a consequence of catalysis because with DA as the substrate, interfacial lipid composition remains invariant. Similar data were obtained if large, unilamellar vesicles were used in place of monolayers (J. M. Muderhwa, W. E. Momsen, and H. L. Brockman, in preparation), if pancreatic triglyceride lipase (PL) replaced CEL in either the monolayer (Muderhwa & Brockman, 1992b) or bilayer system and if other lipase substrates, like diacylglycerol, replaced DA (Cunningham *et al.*, 1989; Tsujita *et al.*, 1989). Also in LE monolayers of DA–POPC, DA above 0.3 mole fraction markedly stimulates the ability of a bacterial phospholipase C to hydrolyze POPC (Tsujita & Okuda, 1992). As for CEL, this activation of phospholipase C mimics that caused by diacylglycerol, the product of the reaction.

These fatty acid effects, together with similar effects of diacylglycerol and other neutral glycerides on lipolytic activities, have led to the hypothesis that an important factor

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DA, 13,16-*cis,cis*-docosadienoic acid; LE, liquid-expanded; PPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; LC, liquid-condensed; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; MPPC, 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PA, palmitic acid; CEL, porcine pancreatic carboxylester lipase; PL, porcine pancreatic triglyceride lipase.

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in the regulation of lipolytic enzymes *in vitro* and *in vivo* is the lateral distribution of phospholipid and non-phospholipid species in the interfacial plane (Muderhwa & Brockman, 1992b). For phospholipase A_2 activation by fatty acids, this takes the form of lateral phase separation of reaction products in both monolayers (Reichert *et al.*, 1992) and bilayers (Jain *et al.*, 1989; Burack *et al.*, 1993). However, in the composition range over which DA–POPC monolayers exhibit the critical transition for lipase-catalyzed oxygen exchange, miscibility could not be unambiguously determined (Smaby & Brockman, 1985, 1992). Similar, equivocal results have been obtained in other monolayer miscibility studies of fatty acids and phosphatidylcholines in the LE state (Lundberg & Ekman, 1979; Torosian & Lemberger, 1968; Finer & Phillips, 1973; Smaby & Brockman, 1985; Casas & Baszkin, 1992). Both miscibility and immiscibility of the components have been concluded from such data. In one study of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PPPC)–oleic acid monolayers, it was noted that the onset surface pressure for the LE to liquid-condensed (LC) phase transition increased monotonically with oleic acid content up to an equimolar mixture (Lewis & Hadgraft, 1990). From this and the reduced cooperativity of the transition, it was concluded that the components displayed "a degree of miscibility" in the LE state.

Whether or not diacylphosphatidylcholines and fatty acids laterally phase separate under the conditions where lipases and phospholipase C show critical transitions in substrate accessibility is important to assess. In the present study we have used perturbation of phase transition surface pressures by either a phospholipid or fatty acid to assess its miscibility with either DA or POPC, respectively, in the LE state. For one system, the accessibility of DA to CEL and PL was also determined. The results show that lateral phase separation in these monolayers is not a necessary condition for observing the critical transition in fatty acid availability to lipases and are relevant to the regulation of lipid-mediated signaling in cells.

MATERIALS AND METHODS

Phospholipids were purchased from Avanti Polar Lipids. DA and palmitic acid (PA) were from NuChek Prep, Inc., Elysian, MN. The purity of the lipids was assessed by thin-layer chromatography and found to be greater than 99%. They were used without further purification for determination of surface pressure–surface potential–molecular area isotherms. CEL and PL were purified to homogeneity as previously described (Cunningham *et al.*, 1989; Rudd *et al.*, 1987). The level of colipase in the PL preparation was 0.56 mol%. Hexane (non-spectro grade) was from Burdick and Jackson Laboratories (Muskegon, MI), ethanol was distilled from zinc and KOH (Perrin *et al.*, 1966) and petroleum ether was purified using a protocol described earlier (Smaby & Brockman, 1981). Water was purified by reverse osmosis, mixed-bed deionization, adsorption on activated charcoal, and filtration through a 0.22-μm Durapore membrane (Millipore Corporation, Bedford, MA). Buffers prepared with this water were degassed under vacuum, filtered through a Diaflo hollow fiber filter with a molecular weight cutoff of 10000 (Amicon Corp., Danvers, MA), and stored under argon until use. All other materials were reagent grade and used without further purification.

Solutions of phospholipids were prepared in petroleum ether/ethanol (95:5) and fatty acids were dissolved in petroleum ether alone. Both were stored at –70 °C until use and no degradation of lipids was detectable by comparison of surface pressure–area isotherms collected before and after the studies of lipid mixtures. Fatty acid concentrations were determined gravimetrically and phosphatidylcholine concentrations were assessed by measuring lipid phosphorus content (Bartlett, 1959). Surface pressure–surface potential–molecular area isotherms for lipids were measured using an automated Langmuir film balance (Brockman *et al.*, 1980; Brockman *et al.*, 1984). Phase transition onset pressures were determined in all but a few cases by an algorithm based on second and third derivatives of surface pressure with respect to area. The remainder were estimated by visual inspection of the isotherm.

The lipase-catalyzed conversion of DA to 93.6 atom % $[^{18}O]_2$DA for use in oxygen-exchange experiments has been recently described (Muderhwa & Brockman, 1992b). Measurements of lipase-catalyzed oxygen exchange were conducted using an automated film balance (Tsujita & Brockman, 1987) with a reaction chamber area of 26 cm^2 over a 27-mL subphase stirred at 100 rpm using a protocol reported earlier (Muderhwa *et al.*, 1992). Following recovery of DA after exposure to the lipase solution, it was hydrogenated, converted to its methyl ester, and analyzed for ^{18}O content and distribution by gas chromatography–mass spectrometry (Schmid *et al.*, 1988; Kuwae *et al.*, 1990; Muderhwa *et al.*, 1992).

RESULTS

For LE monolayers formed from mixtures of phosphatidylcholines and either fatty acids or simple glycerides, there typically exists a range of compositions over which (i) the collapse surface pressure of the monolayer is essentially constant, (ii) average molecular area–composition plots are nearly linear at all surface pressures in the LE state and (iii) surface pressure-independent criteria of miscibility also display ideal/immiscible composition dependencies (e.g., Smaby & Brockman, 1985, 1992). Figure 1 illustrates this behavior for mixtures of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) and DA at pH 6.6, 24 °C. Panel a shows that up to a DA mole fraction of 0.67, the collapse pressure of the monolayer is approximately constant at 46–47 mN/m, precluding reliable determination of miscibility. Specifically, the data are consistent with either miscibility or with formation of a 2:1 complex of SOPC and DA which collapses at the same surface pressure as SOPC and which is immiscible with SOPC. At mole fractions of DA above 0.67, the collapse transition onset pressure of the films is clearly composition dependent, indicating miscibility of POPC in the DA-rich monolayers. The second diagnostic parameter widely used to assess miscibility is the composition dependence of the average molecular area of the monolayer components at selected surface pressures. As shown in Figure 1, panel b, these show little deviation from additivity at surface pressures of 1, 11, and 21 mN/m, again precluding reliable determination of miscibility state between 0.0 and 0.67 DA. The same is true for four miscibility parameters (Figure 1, panels c–f) which for each lipid mixture are determined from data at all surface pressures in the LE state (Smaby & Brockman, 1992). These are the dehydrated cross-sectional area of the lipids (panel c), the activity coefficient of interfacial water (panel d), the surface dipole moment (panel e), and the area-independent surface potential (panel f). In summary, neither the phase diagram nor any of the five descriptive parameters available allow the existence of liquid–liquid immiscibility to be reliably differentiated from ideal miscibility up to 0.67 mole fraction of DA.

The behavior shown in the six panels of Figure 1 is typical for mixtures comprised of a phosphatidylcholine and a fatty

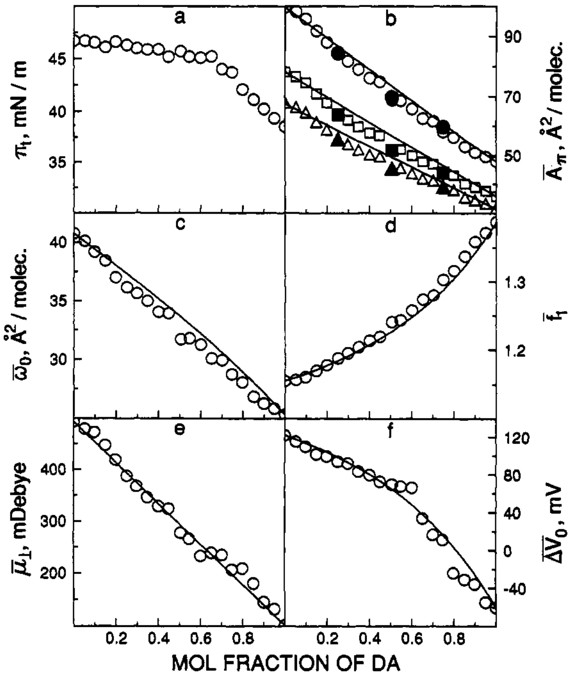

FIGURE 1: Miscibility analysis for SOPC–DA mixed monolayers. (a) Phase transition pressures, π_t, defining the upper limit of the LE phase. (b) Average molecular areas, $\bar{A}_\pi$, obtained by evaluating fitted isotherms at surface pressures of 1 (○), 11 (□) and 21 (△) mN/m. For comparison, filled symbols show experimental data points from isotherms obtained at selected mole fractions. The solid lines show ideal behavior. (c) Fitted values of the experimental, $\bar{\omega}_0$, (symbols) and ideal (line) dehydrated molecular area. (d) Fitted values of the experimental, $\bar{f}_1$, (symbols) and ideal (line) water activity coefficient. (e) Fitted values of the experimental, $\bar{\mu}_\perp$, (symbols) and ideal (line) surface dipole moment. (f) Fitted values of the experimental, $\Delta\bar{V}_0$, (symbols) and ideal (line) area-independent surface potential.

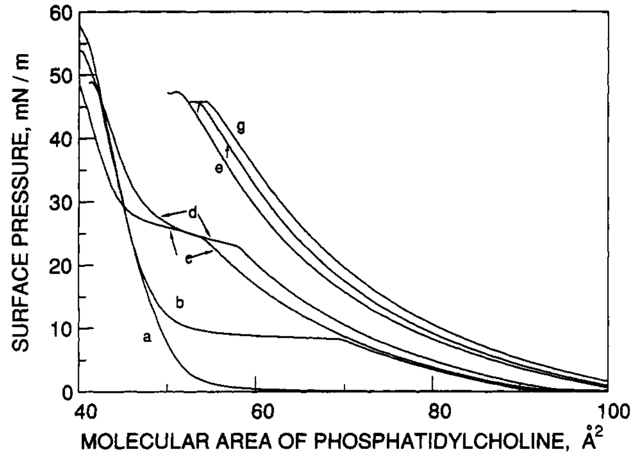

FIGURE 2: Surface pressure–molecular area isotherms for phospholipid monolayers at pH 6.6. (a) PPPC, 15 °C; (b) PPPC, 24 °C; (c) MPPC, 24 °C; (d) PPPC, 32 °C; (e) SOPC, 15 °C; (f) SOPC, 24 °C; (g) SOPC, 32 °C.

acid each of which, alone, forms a LE monolayer which collapses directly to a three-dimensional phase without passing through any more-condensed monolayer states. Figures 2 and 3, curves e–g, show that such surface pressure–area isotherms are obtained with either DA or the SOPC homolog, POPC, at experimental temperatures of 15, 24, and 32 °C. Isotherms obtained with POPC monolayers at 24 °C but with subphases having a pH of either 5.6 or 7.6 (not shown) were essentially identical to that shown for pH 6.6 and 24 °C (curve f) as expected from the zwitterionic nature of the phosphorylcholine group.

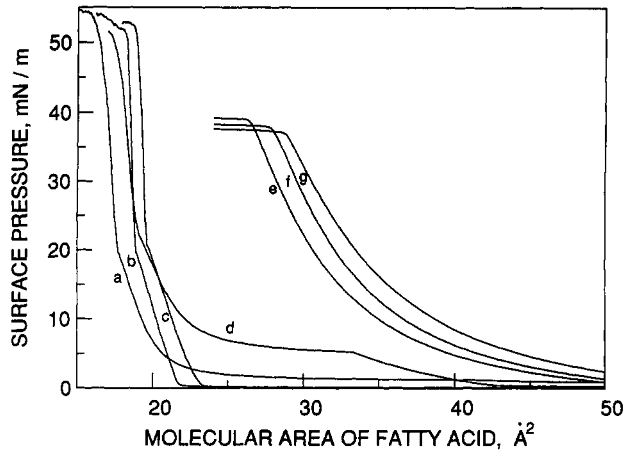

FIGURE 3: Surface pressure–molecular isotherms for fatty acid monolayers. (a) PA, pH 7.6, 24 °C; (b) PA, 15 °C; (c) PA, pH 5.6, 24 °C; (d) PA, pH 6.6, 32 °C; (e) DA, pH 6.6, 15 °C; (f) DA, pH 6.6, 24 °C; (g) DA, pH 6.6, 32 °C.

To overcome the limitations of the analysis described above for data obtained with mixtures of such *cis*-unsaturated lipids, substitutions were made in their aliphatic compositions to increase cohesive interactions among the chains. The surface pressure–area isotherm for a film of PPPC at 24 °C (Figure 2, curve b) is more condensed at all pressures than that of POPC. In addition to a poorly-defined monolayer collapse at a surface pressure above 50 mN/m, the PPPC isotherm also exhibits the well-characterized transition from the LE state to the LC state as molecular area is decreased. The onset of this transition occurs at a surface pressure, π_0, of 7–8 mN/m at 24 °C. As shown in Figure 2 and described earlier (e.g., Phillips & Chapman, 1968), π_0 is highly temperature-dependent, increasing to about 23 mN/m when the isotherm is obtained at 32 °C (Figure 2, curve d). At 15 °C the LE–LC transition of PPPC cannot be identified and the film appears to pass directly from the gaseous to the LC state (Figure 2, curve a). Isotherms obtained at 24 and 32 °C with 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (PSPC), which has two additional CH_2 groups in the saturated acyl moiety at position 2 of glycerol, are similar, but slightly more expanded, than those for PPPC at 15 and 24 °C, respectively (data not shown). Similarly, replacing PPPC with 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (MPPC), which has a two-carbon shorter, saturated fatty acid at the 1 position of glycerol, increases π_0 from 7–8 mN/m (Figure 2, curve b) to 25 mN/m (curve c). Thus, as noted earlier for diacyl phosphatidylcholines with identical acyl groups (Phillips & Chapman, 1968), increasing or decreasing the length of the aliphatic moiety at either position of the glycerol moiety is equivalent to changing the experimental temperature.

Results comparable to those shown in Figure 2 are obtained when the 22-carbon, di-*cis*-unsaturated fatty acid, DA, is replaced by the 16-carbon, saturated fatty acid, palmitic acid (PA). Whereas isotherms obtained with monolayers of DA at 15, 24, and 32 °C are relatively expanded and collapse directly from the LE state (Figure 3, curves e–g), those obtained with PA (curves a–d) show more complex behavior. For PA at 32 °C and pH 6.6 the LE–LC transition is evident at 6 mN/m (Figure 3, curve d). At 15 °C the PA isotherm shows a transition from the gaseous directly to the LC state ending at about 22 Å^2 per molecule and, at a surface pressure of 20 mN/m, the beginning of a transition to a more condensed state before the film collapses above 50 mN/m (Figure 3, curve b). The isotherm for PA obtained at 24 °C completes the gaseous to LC transition at about 23 rather than 22 Å^2/

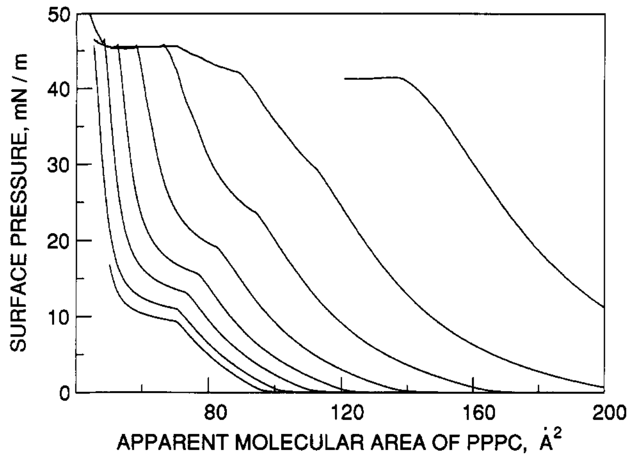

FIGURE 4: Surface pressure–molecular area isotherms for PPPC–DA mixtures. Mole fractions of DA left to right: 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80.

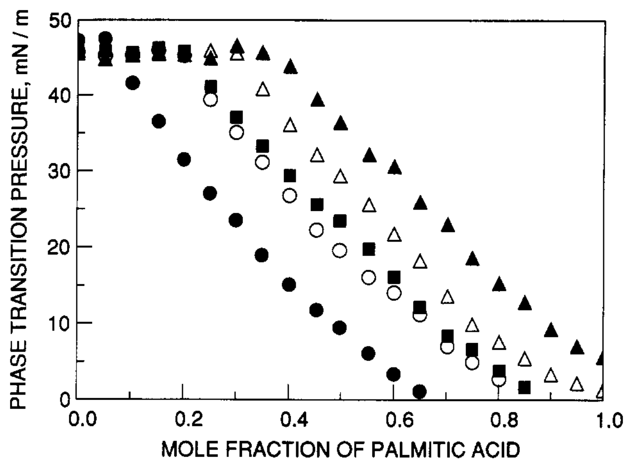

FIGURE 5: Partial phase diagrams for POPC–PA mixtures. Phase transition pressures defining the upper limit of the LE state (π_t) were determined as described in the text. (●) pH 6.6, 15 °C; (○) pH 5.6, 24 °C; (■) pH 6.6, 24 °C; (△) pH 7.6, 24 °C; (▲) pH 6.6, 32 °C.

Table 1: Miscibility Ranges for Diacylphospholipid–Fatty Acid Mixtures Determined from Phase Transition Pressures

fatty acid	phospholipid	pH	*T*, °C	mole fraction range
DA	MPPC	6.6	24	0.00–1.00
DA	PPPC	6.6	15	0.20–0.80
DA	PPPC	6.6	24	0.00–0.80
DA	PPPC	6.6	32	0.00–0.75
PA	POPC	5.6	24	0.25–0.80
PA	POPC	6.6	24	0.25–0.85
PA	POPC	7.6	24	0.35–0.95
PA	POPC	6.6	15	0.10–0.65
PA	POPC	6.6	32	0.40–1.00
DA	PSPC	6.6	24	0.00–0.75
DA	PSPC	6.6	32	0.00–0.70

molecule but is nearly identical to the 15 °C isotherm at surface pressures of 10 mN/m and higher (not shown). Lowering the subphase pH to 5.6 with PA at 24 °C yields an isotherm (Figure 3, curve c) which "lifts off" at about 23 Å^2 but parallels that of PA at 15 °C and pH 6.6. However, raising the pH to 7.6 at 24 °C induces an LE–LC transition with a π_0 of 1.2 mN/m at a molecular area of 42.5 Å^2 (Figure 3, curve a).

In addition to aliphatic chain length, *cis* unsaturation, and experimental temperature, the onset pressure for the LE to LC transition, π_0, may also be altered by changes in lipid composition. This is shown in Figure 4 for mixtures of PPPC and DA. To improve clarity, the isotherms are plotted relative to the apparent molecular area of PPPC. At 0.1 mole fraction of DA (Figure 4, left isotherm), π_0 is 2–3 mN/m higher than for PPPC alone (Figure 3, curve b). As the mole fraction of the more fluid DA is increased from 0.1 to 0.8 in 0.1 increments (left to right in Figure 4), π_0 increases monotonically to near 30 mN/m. At 0.8 mole fraction of DA (far right curve, Figure 4) the transition cannot be detected. For such two-component monolayers at constant temperature and bulk pressure, a composition-dependent increase in π_0 indicates that the lipids are miscible in the LE state over the range the transition onset pressure is composition dependent (Crisp, 1949). Thus, as reported previously (e.g., Lewis & Hadgraft, 1990; Ali *et al.*, 1991), the lipid composition dependence of π_0 can be used to assess lipid miscibility in the LE state of monolayers. Specifically, over the compositional range in which π_0 increases with increasing content of a second lipid which forms only LE monolayers under the conditions used, the lipids are miscible in the LE state. Conversely, were lateral phase separation to occur, π_0 for the phase exhibiting the LE–LC transition should remain constant as the content of the more fluid lipid is increased. The same logic applies if the LE state collapses to a bulk phase, rather than the LC state, upon further compression.

For other mixtures prepared from pairs of the phosphatidylcholines and fatty acids discussed above, families of surface pressure–molecular area isotherms, like those shown in Figure 4, were determined (not shown). Each set of binary lipid mixtures for which isotherms were obtained consisted of either PA and POPC or DA and either MPPC, PPPC, or PSPC. All mixtures were investigated at 24 °C and a subphase pH of 6.6. To determine if the observed phase behavior of the mixtures was limiting behavior, some mixtures were also characterized at 15 and 32 °C or at pH values of 5.6 and 7.6. From the isotherms obtained, phase transition pressures were identified as described in Methods. The results are summarized in Figures 5, 6, and 7a as partial phase diagrams. For each isotherm these show only the transition surface pressure, π_t, at which the LE state, if formed, begins the transition to either a more condensed monolayer state or to a collapsed phase. Thus, the data in the figures defines the upper pressure limit of the LE phase(s) as a function of monolayer lipid composition.

Figure 5 shows data obtained with mixtures of PA and POPC. Under each set of conditions used, a compositional range exists over which the LE state is formed and π_t changes with lipid composition. For example, at 15 °C and pH 6.6 (filled circles) the range extends from PA mole fractions ≥0.05 to ≤0.65. These miscibility ranges are summarized in Table 1. It should be noted that for compositions outside the range over which π_t is composition-dependent, immiscibility is not implied; rather, miscibility state simply cannot be determined using this approach. Increasing the temperature shifts the range from lower to higher mole fractions of PA, as does increasing the pH at 24 °C. Note that neither the upper nor lower limit of the miscibility range suggests any particular stoichiometry for the interactions which lead to the observed behavior. Similar results are obtained if DA is mixed with either PPPC or PSPC at different temperatures (Figure 6). In this case, however, note that the upper limit of the miscibility range based on the LE–LC transition, i.e., the range for which $\pi_t = \pi_0$, shows an upper limit around 0.8 mole fraction of DA. At higher DA mole fractions π_0 cannot be detected and π_t changes slightly with DA composition suggesting miscibility in this range as well. Shown in Figure 7a (circles) are π_t values for MPPC and DA at 24 °C, pH 6.6. These are similar

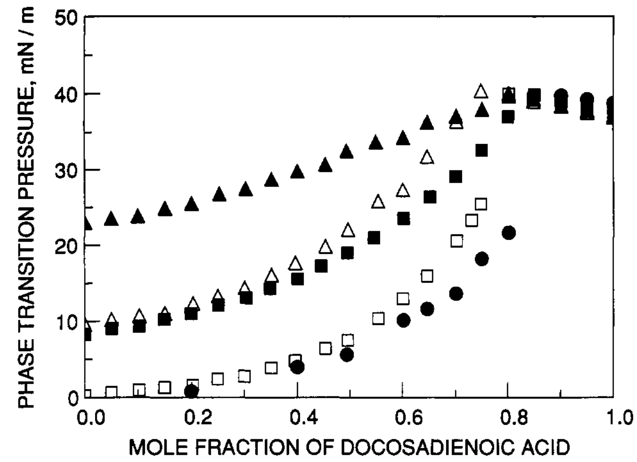

FIGURE 6: Partial phase diagrams for phosphatidylcholine–DA mixtures. Phase transition pressures defining the upper limit of the LE state were determined as described in the text. (▲) PPPC, 32 °C; (△) PSPC, 32 °C; (■) PPPC, 24 °C; (□) PSPC, 24 °C; (●) PPPC, 15 °C.

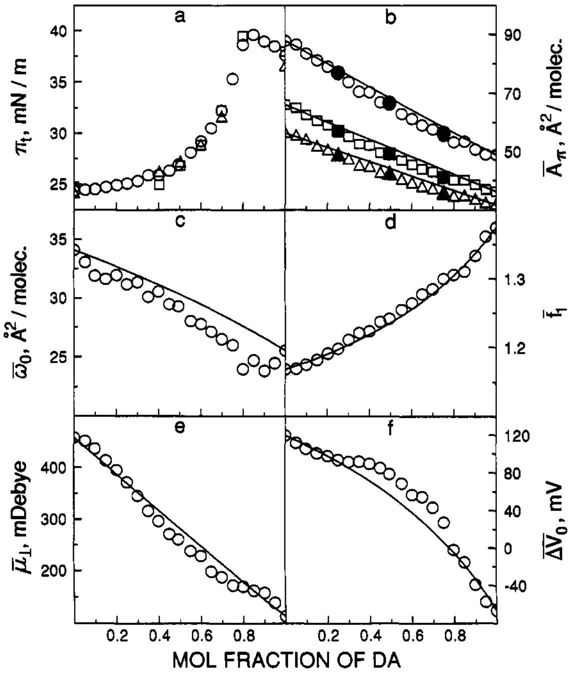

FIGURE 7: Miscibility analysis for DA–MPPC mixed monolayers. (a) Phase transition pressures, π_t, defining the upper limit of the LE phase (○). π_t values obtained after annealing for 1 h at 0 mN/m (△) or 20 mN/m (□). (b) Average molecular areas, $\bar{A}_\pi$, obtained by evaluating fitted isotherms at surface pressures of 1 (○), 11 (□), and 21 (△) mN/m. For comparison, filled symbols show experimental data points from isotherms obtained at selected mole fractions. The solid lines show ideal behavior. (c) Fitted values of the experimental, $\bar{\omega}_0$, (symbols) and ideal (line) dehydrated molecular area. (d) Fitted values of the experimental, $\bar{f}_1$, (symbols) and ideal (line) water activity coefficient. (e) Fitted values of the experimental, $\bar{\mu}_\perp$, (symbols) and ideal (line) surface dipole moment. (f) Fitted values of the experimental, $\Delta\bar{V}_0$, (symbols) and ideal (line) area-independent surface potential.

to the data shown in Figure 6 in that the LE–LC transition is not detected above 0.8 mole fraction of DA and that miscibility is suggested over the entire range of compositions.

The π_t values presented in Figures 5–7a were all obtained from surface pressure–area isotherms obtained by continuous compression of the lipid film from larger to smaller areas. Although the compression rate was kept at ≤4 Å^2 per lipid molecule per minute, the possibility exists of kinetically trapping a particular lateral distribution of the molecules during film compression (e.g., Cadenhead *et al.*, 1976). If it occurs in either the gaseous or LE state, it can lead to the π_t data reflecting the properties of this nonequilibrium monolayer state. To test this possibility, control experiments were performed in which films of MPPC and DA at selected compositions were allowed to anneal at essentially constant area for a period of 1 h at a surface pressure of approximately 0 before being compressed or after being compressed to 20 mN/m. The π_t values obtained are also shown in Figure 7a. Their near identity with those obtained by continuous compression indicates that no significant change in lipid lateral distribution, especially phase separation, occurred during the incubations at either high or low surface pressure. This strongly suggests that the miscibility ranges indicated in Figures 5–7a and summarized in Table 1 represent near equilibrium behavior of the system.

Films comprised of DA, MPPC, or their mixtures exhibit π_t values of 25 mN/m or higher (Figure 7a). Thus, the LE state exists over a range of surface pressure sufficient to apply the type of miscibility analysis shown in Figure 1 for the DA–POPC system. The results are shown in panels b–f of Figure 7 and are presented using the same scale factors used in Figure 1. Comparison of the figures reveals only small deviations of the parameters from ideal behavior. The degree of mixing nonideality shown in panel b of both figures is essentially the same, only a few percent at most. The same is true of the water activity coefficients (panel d) for each set of mixtures which exhibit nearly ideal behavior in each case. The DA–MPPC system shows a slightly greater degree of nonideality in the lipid dehydrated areas (panel c), especially in the compositional range around 0.8 mole fraction of DA where the phase diagram shows eutectic behavior. The surface potential-derived parameters shown in panels e and f are also slightly less ideal than those for the DA–SOPC system, especially around a 1:1 mole ratio. Again, however, the changes are relatively small. Overall, the data in panels b–f of Figure 7 are consistent with the LE state miscibility of DA and MPPC indicated by panel a and show that it deviates only slightly from ideal behavior.

A second reason for the more detailed characterization of the DA–MPPC system shown in Figure 7 was that mixtures of these two miscible lipids could be used at 24 °C to test for the existence of a critical transition in DA availability to lipases. Their suitability for this derives from the π_t values of all DA–MPPC mixtures being >20 mN/m (Figure 7a). This requirement is not absolute, but being able to measure substrate susceptibility to PL and CEL at monolayer surface pressures >20 mN/m minimizes potential contributions of lipase surface denaturation to the results (Tsujita & Brockman, 1987). Using the same protocol applied earlier to DA–POPC films (Muderhwa & Brockman, 1992a; Muderhwa & Brockman, 1992b), the lipase-catalyzed exchangeability of oxygen between [^{18}O]$_2$-DA in LE monolayers of MPPC and subphase water at 24 °C and pH 6.6 was measured. Shown in Figure 8 are the extents of [^{18}O]$_2$DA carboxyl oxygen atoms exchanged during 10 min following addition of CEL lipase (30 nM) or PL (10 nM) in the aqueous subphase. In each case the extent of exchange is about 10% until the [^{18}O]$_2$DA mole fraction exceeds 0.45, above which exchange increases abruptly to near 100%. Comparable results were obtained when the enzyme concentrations were reduced 10-fold except that above 0.45 mole fraction of [^{18}O]$_2$DA, exchange was less than complete, reaching 80% with CEL and 70% with PL (data not shown). These results are nearly identical to those obtained with [^{18}O]$_2$-DA–POPC monolayers, particularly with respect to the

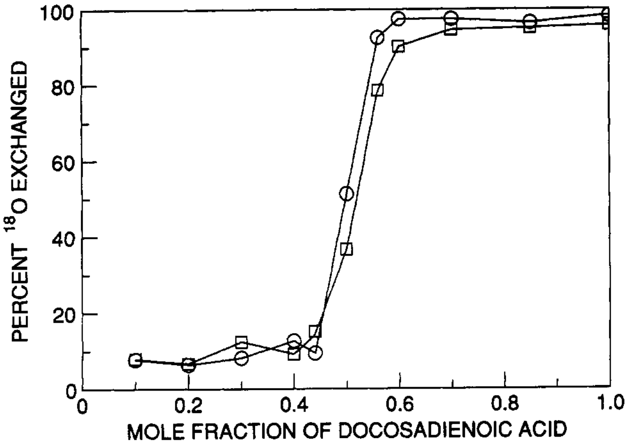

FIGURE 8: Lipid composition dependence of lipase-catalyzed ^{18}O exchange in mixed monolayers of ^{18}O-enriched DA and MPPC. Bulk phase CEL concentration was 30 nM (○) and PL concentration was 10 nM (□).

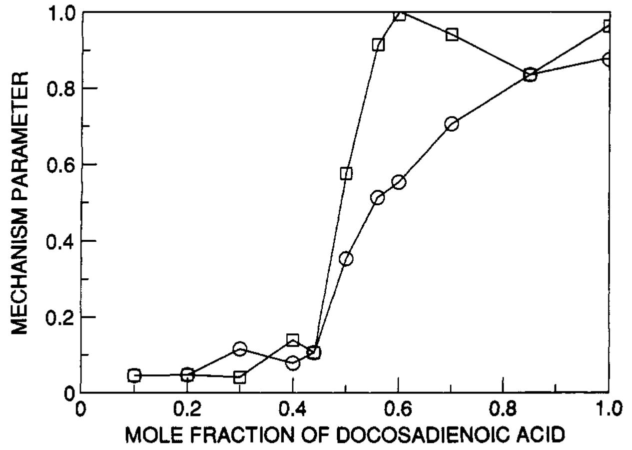

FIGURE 9: Lipid composition dependence of the mechanism parameter for lipase-catalyzed ^{18}O exchange in DA–MPPC monolayers. Parameter values were calculated from the data of Figure 8 as described in the text. CEL (○), PL (□).

apparently critical change in extent of lipase-catalyzed oxygen exchange which occurs between 0.45 and 0.55 mole fraction of $[^{18}O]_2DA$.

Another feature of the DA–POPC system studied earlier is that the critical transition in oxygen exchange is accompanied by a similar, abrupt change in a mechanism parameter calculated from the distribution of ^{18}O-containing DA species following exposure to a lipase. As defined earlier, the value of that parameter is 0.0 for a coupled or a concerted exchange mechanism and 1.0 for a random, sequential mechanism (Muderhwa *et al.*, 1992). Values of the mechanism parameter calculated from the distribution of ^{18}O in DA for the experiments of Figure 8 are shown in Figure 9. These are low at low mole fractions of $[^{18}O]_2DA$ and increase to values near 1.0 at $[^{18}O]_2DA$ mole fractions above 0.45. In the case of the data obtained with CEL, the increase in the parameter values is not quite as abrupt as observed earlier with $[^{18}O]_2DA$–POPC monolayers (Muderhwa & Brockman, 1992a) or with PL in the present or the earlier (Muderhwa & Brockman, 1992b) study. Irrespective of this small difference, the mechanism parameter clearly shows discontinuous behavior as $[^{18}O]_2DA$ mole fraction exceeds 0.45.

DISCUSSION

This study was undertaken to determine if lateral lipid phase separation was responsible for the composition-dependent stimulation of lipases and phospholipase C which occurs as fatty acid reaches and exceeds approximately equimolar concentration in mixtures with phosphatidylcholine. For the particular fatty acid and phosphatidylcholine, DA and POPC, used initially to define the phenomenon, miscibility state could not be assessed. Similarly, results from earlier monolayer studies involving related fatty acids and phosphatidylcholines were inconclusive and contradictory. Also, as with previous studies of hydrated bulk dispersions of phosphatidylcholines and fatty acids (e.g., Cevc *et al.*, 1988), the pH of the aqueous phase, its ionic strength, and its divalent metal ion concentration generally did not match that of the lipase or phospholipase C studies. Therefore, in this study, phosphatidylcholine–fatty acid miscibility in the LE state was determined under conditions where two, genetically-distinct lipases of pancreatic origin have been shown to exhibit the critical transition in substrate accessibility. To enable miscibility to be assessed in the present study, either the fatty acid, DA, or the phosphatidylcholine, POPC, used earlier with the lipases was replaced by one which could undergo the LE–LC transition. For such binary lipid mixtures, the composition-dependence of the onset pressures for the phase transitions which define the compression limit of the LE phase, π_t, were used to determine the minimum range of component miscibility in the LE state. Variations in pH and temperature were made to determine if the miscibility characteristics determined at pH 6.6 and 24 °C were limits.

Each mixture examined displayed a range of compositions over which miscibility of phosphatidylcholine and fatty acid could be demonstrated (Table 1). Control experiments with the MPPC–DA system suggest further that this miscibility is not a metastable phenomenon which results from kinetic trapping of a particular lateral distribution of lipid molecules during film compression. Considering the data in aggregate, the demonstrable miscibility ranges encompass the entire range of lipid compositions and suggest that the observed miscibility is not unique to a pH value of 6.6 or a temperature of 24 °C. Considering the mismatch in chain lengths and in chain saturation between MPPC and DA as compared to SOPC and DA, the minimal nonideality shown by the MPPC–DA system (Figure 7) is surprising. It suggests that as long as the lipids are in the LE state, chain structure is not an important determinant of miscibility. Overall, the general occurrence of nearly ideal LE state miscibility of DA and phosphatidylcholines with saturated chains and of POPC–PA mixtures at different temperatures and pH values strongly suggests that DA and POPC in monolayers are miscible at all compositions. If so, the critical transition in DA availability to lipases in such monolayers would not require lateral phase separation.

Although the miscibility measurements strongly support the absence of lateral phase separation in the compositional range of the critical transition in lipase activity, the miscibility and reactivity measurements were made with monolayers of different lipids. To address more directly the relationship between miscibility and enzyme activity, measurements of ^{18}O exchange between the carboxyl group of DA and water were made in MPPC monolayers. The $[^{18}O]_2DA$–MPPC system shows a clear dependence of π_0 on $[^{18}O]_2DA$ mole fraction up to 0.8, demonstrating miscibility over this compositional range. With either CEL or PL, this system also exhibits a critical transition in $[^{18}O]_2DA$ availability to lipase. Not only are the composition dependencies of exchange essentially identical to those obtained with POPC and $[^{18}O]_2$DA (Figure 8), but also they are accompanied by apparent shifts in the reaction mechanism (Figure 9). These phenomena are the hallmarks of the critical transition observed with the $[^{18}O]_2$DA–POPC system (Muderhwa & Brockman, 1992a,b). Thus, lateral phase separation is not required for the critical transition to occur.

The nearly ideal mixing of fatty acid and phosphatidylcholine in LE monolayers in the range of the critical transition in fatty acid accessibility to lipases implies that the arrangement of the lipids in the interface is essentially random. Thus, at mole fractions of fatty acid well below that of the critical transition range the fatty acid substrate is dispersed as a statistical distribution of individual molecules and clusters in a continuum of phosphatidylcholine. Such an essentially random distribution is consistent with the limited extent of lipase adsorption, the limited fraction of fatty acid molecules accessible to the lipases, and the apparently coupled or concerted mechanism indicated by the low values of the mechanism parameter for fatty acid mole fractions below the critical transition range (Muderhwa & Brockman, 1992a,b). Also consistent with this picture are the measured upper limits of the average size of fatty acid domains to which lipases adsorb as the lipid composition changes. At 0.35 mole fraction of DA in POPC monolayers the value was about 1700 molecules of DA. At 0.45 mole fraction of DA, just below the range of the critical transition, the value increased to 10 000 (Muderhwa & Brockman, 1992a,b). According to the proposed model (Muderhwa & Brockman, 1992b), the critical transition in accessibility of substrate to lipases coincides with the change of the surface continuum from phosphatidylcholine to fatty acid. Hence, in the range of the critical transition the statistically predicted fatty acid cluster size becomes infinite. At fatty acid mole fractions above the critical transition lipid miscibility implies that the phosphatidylcholine should be randomly distributed, just as is fatty acid below the transition. This is consistent with the apparent affinity of CEL for films of fatty acid and phosphatidylcholine (2:1) being 10–20 fold lower than for fatty acid alone (Tsujita *et al.*, 1987). Thus above, as well as below, the range of the critical composition the miscibility characteristics of fatty acid–phosphatidylcholine mixtures demonstrated in this study supports the proposed model of lipase regulation at such interfaces (Muderhwa and Brockman, 1992b).

The lipase-catalyzed critical transition in oxygen exchangeability has also been observed with liquid-crystalline bilayers of SOPC and $[^{18}O]_2$DA (J. M. Muderhwa and H. L. Brockman, in preparation). Although miscibility of fatty acids and phosphatidylcholines above the chain melting transition has generally been concluded from physical studies of bilayers, some reports suggest that a 1:2 complex of phosphatidylcholine and protonated fatty acid may cluster (Koynova *et al.*, 1987; Cevc *et al.*, 1988) or even laterally phase separate (Ortiz & Gómez-Fernández, 1987). Monomolecular films in the LE state are highly analogous to lamellar, liquid-crystalline bilayers (e.g., Nagle, 1980; MacDonald & Simon, 1987; Vaknin *et al.*, 1991). Thus, the nearly ideal miscibility of fatty acids and phosphatidylcholines demonstrated in this study would suggest that lateral phase separation is not a prerequisite for the critical transition in DA oxygen exchange in bilayers. Complicating this extrapolation, however, is the ability of fatty acids to induce mesomorphic transitions in hydrated dispersions above the chain melting temperature (e.g., Koynova *et al.*, 1988).

The lack of a requirement for lateral phase separation for induction of lipase activity appears to differ from the product-induced activation of phospholipases A_2. The connection between accumulation of a reaction products of phosphatidylcholine hydrolysis and the end of a latency period in phospholipolysis rate was established by Jain and co-workers (Jain *et al.*, 1989) and is frequently referred to as being due to lateral phase separation (e.g. Burack *et al.*, 1993). It is clear that macroscopic, lateral phase separation of reaction products will occur under the conditions of high pH and Ca^{+2} often used to study phospholipolysis (e.g. Maloney & Grainger, 1993). However, as recently discussed by Burack *et al.* (1993) activation of phospholipase can also occur at low Ca^{+2} concentrations if products accumulate into enriched domains which are still partially miscible with phosphatidylcholine. This points out a source of confusion in comparing lipase and phospholipase studies, namely, that lateral phase separation represents a continuum of behavior between ideal miscibility and macroscopic phase separation. Thus, it is likely that phospholipases A_2 respond to lateral lipid distribution in the same manner as lipases. If so this phenomenon is of potential physiological importance because in the intestine pancreatic phospholipase A_2 as well as the pancreatic lipases used in this study function at pH values between 5.5 and 7.5 where free fatty acids carry little or no charge (e.g., Hernell *et al.*, 1990) and Ca^{+2} binding to fatty acids is negligible (Hauser *et al.*, 1979).

It has been demonstrated earlier that physiologically relevant ester substrates of lipases, like diacylglycerols, exhibited the critical transition in substrate accessibility to lipases as their mole fraction was increased (Tsujita *et al.*, 1989). This activation occurred in a compositional range over which the ester substrate and phosphatidylcholine were clearly miscible (Smaby & Brockman, 1985). However, it was uncertain if hydrolysis products, like fatty acid, generated by the lipases induced lateral phase separation which, in turn, led to the critical phenomenon. This was addressed by using fatty acid as a substrate, as in the present study, so that lipid composition in the interface remained invariant. However, the extent of miscibility of fatty acid and phosphatidylcholine has not, until now, been determined. Together these studies show that activation of lipase activity can occur within a single surface phase for both fatty acid and ester substrates. Unless lipases are considered unique, the import of this work is that the lateral distribution of phospholipid and non-phospholipid molecules in a single surface phase can be an almost all-or-none regulator of enzyme activities in interfaces.

Certainly, the global concentrations of diacylglycerols or fatty acids in membranes or cells do not reach the 50 mol % required for observation of the critical activation of lipases. However, sufficiently rapid generation of these reaction products or the presence of diffusional barriers in the vicinity of a membrane-bound lipolytic enzyme would likely generate local concentrations of reaction products exceeding their critical concentration relative to membrane phospholipids. This event could then trigger the translocation of cytoplasmic enzymes like protein kinases C and their ultimate activation. Because such product generation would be localized and of a transient nature, it can be termed the *topo-temporal* regulation of interfacial reactions. A major challenge for future studies is to determine if such a mechanism can be demonstrated *in vitro* or *in vivo*.

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