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Positive Surface Charge Inhibition of Phospholipase A_2 in Mixed Monolayer Systems[1]

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Inhibition of pancreatic phospholipase A_2 by surface-active local anesthetics was recently reported by this laboratory to be due to enzyme-anesthetic interaction in the subphase and surface effects. In order to study surface effects in the absence of subphase effects, a long-chain tetracaine analog which was completely insoluble in the subphase, dimethylaminoethyl *p*-decoxybenzoate, was synthesized. To determine if inhibition was due to the positive surface charge of the analog or some other effect related to structure, the analog's inhibitory effects were compared with those of octadecylamine. Analog-didecanoyl lecithin (PC) monolayers showed nonideal mixing as evidenced by a condensing effect, while octadecylamine-didecanoyl PC monolayers showed ideal mixing. The apparent pK'_a of octadecylamine-dioctanoyl PC micelles (1:4) was 9.9, while that of the analog-dioctanoyl PC micelles (1:4) was 7.6. At pH values where both amines were fully protonated, inhibition of both porcine pancreatic and *Crotalus adamanteus* phospholipase A_2 on the mixed films was maximal and similar (94-97%). Inhibition decreased with increasing pH and decreasing surface charge on both mixed films and at pH values where both amines were 50% protonated, inhibition was half-maximal. At pH 8.5, where the analog was unprotonated, no inhibition was observed. Thus, inhibition of phospholipase A_2 appears to be due to a positive surface charge alone rather than any effects related to anesthetic structure or spacing in the monolayer.

Interest in the effects of charged interfaces on the phospholipases has continued since the early work of Bangham and Dawson (1). The results of many early studies were complicated by the use of impure enzymes and lipid monolayer systems which were not well characterized. Utilizing pure phospholipases and synthetic short-chain phospholipid monolayer systems, this study seeks to determine the effects of a positive surface charge on the kinetics of phospholipase A_2.

Because of their ready penetration into lipid interfaces, cationic surface-active local anesthetics produce a positively charged interface. Hendrickson (2) showed that the degree of penetration of different anesthetics into lecithin monolayers correlated with the degree of anesthetic potency. Several years ago, Scherphof *et al.* (3) and Waite and Sisson (4) showed that these procaine-like anesthetics inhibited phospholipase A_2 in liposomal, mitochondrial, and other membrane systems. Recently, Hendrickson and van Dam-Mieras (5) demonstrated that the least surface-active local anesthetics, lidocaine and procaine, inhibit pancreatic phospholipase A_2 action by interaction with the enzyme in the bulk phase. The more surface-active local anesthetics, dibucaine and tetracaine, might also interact with the enzyme in the bulk phase, although no experimental evidence for this exists. However, since these surface-active anesthetics penetrate readily into the substrate interface, they may inhibit by surface effects at concentrations where bulk phase effects are minimal.

The purpose of this study was to further investigate the nature of the inhibition of

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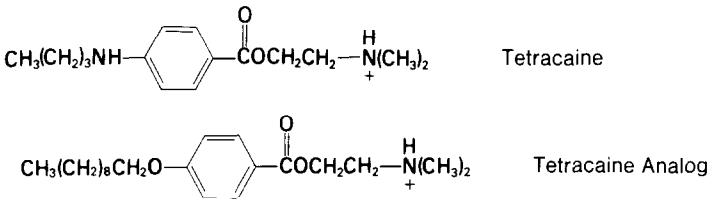

Scheme I. Structures of tetracaine and tetracaine analog.

porcine pancreatic and *Crotalus adamanteus* phospholipase A_2 by cationic surface-active local anesthetics, using tetracaine as a model. In order to completely eliminate subphase effects, a long-chain tetracine analog, dimethylaminoethyl *p*-decoxybenzoate (Scheme I), was synthesized. Since this compound was completely insoluble in the subphase, surface effects could be independently studied. Octadecylamine was used to compare the inhibitory effects of a simple amine with those of the more complex head group structure of the anesthetic to determine if inhibition of phospholipase A_2 is due to surface charge alone or some other effect related to anesthetic structure.

EXPERIMENTAL PROCEDURES

Materials. The zymogen of pancreatic phospholipase A_2 was isolated from porcine pancreas by the method of Nieuwenhuizen *et al.* (6). The zymogen was activated with trypsin as described by Pietersen *et al.* (7). Stock solutions of the pancreatic enzyme (10 mg/ml) were stored at 4°C. *C. adamanteus* phospholipase A_2 (β form) was isolated from the venom as described by Wells (8). Enzyme activity was assayed titrametrically using a pH-stat (6). Stock solutions of this enzyme (10 mg/ml) were stored at 4°C. Both enzymes gave single bands after polyacrylamide gel electrophoresis. Synthetic didecanoyl PC[3] was obtained from Supelco, Inc., Bellefonte, PA. Dioctanoyl PC was synthesized as described by Jensen and Pitas (9). Stock solutions of didecanoyl PC in benzene (1 mM) were prepared and stored at −20°C. The tetracaine analog was synthesized by a Williamson ether reaction between decylchloride and *p*-hydroxybenzoic acid to form *p*-decoxybenzoic acid, followed by acylation with *N,N*-dimethylethanolamine, according to Vanderhaeghe, *et al.* (10). After recrystallization from hexane-ether, the analog gave a single spot with silica gel TLC using chloroform:diethylamine (97:3) as the solvent. The melting point of the analog was 130–132°C. Infra-red and nmr spectra were consistent with structure. Octadecylamine acetate was prepared from the free base and crystallized twice. Stock solutions (1 mM) of the tetracaine analog in benzene and octadecylamine acetate in benzene: methanol (24:1) were stored at 4°C. Analog-didecanoyl PC and octadecylamine-didecanoyl PC mixtures were made from the stock solutions and stored at 4°C. All monolayers were spread over 5 mM Tris-chloride (or Tris-acetate at pH <7) buffer with 5 mM $CaCl_2$ and 0.1 M NaCl (pH 7.0, except where indicated). All solutions were made with doubly distilled water.

Methods. Monolayer compression isotherms were obtained by driving a mobile barrier at a rate of 1.71 cm/min across a single compartment Teflon trough (16.1 × 27.1 cm). Surface pressure was measured by the Wilhelmy method using a platinum plate attached to a Cahn RTL electrobalance. The temperature was maintained between 22–24°C.

Monolayer enzyme reactions were followed using the "zero order trough" as described by Verger and de Haas (11). The monolayers were spread over the buffer subphase (5 mM Tris, 5 mM $CaCl_2$, 0.1 M NaCl) from benzene solutions of didecanoyl PC or didecanoyl PC-amine mixtures to a surface pressure of 10 mN/m (1 mN/m = 1 dyne/cm) (or 7 mN/m where indicated). Then 100 μl of enzyme solution was added, with stirring, under the monolayer in the enzyme compartment. Reactions were followed for approximately 20 min after the recorded plots became linear. Controls of pure didecanoyl PC were run between each experiment with a didecanoyl PC-amine mixture and all experimental rates were expressed relative to these control rates. Slow adsorption of the enzyme to the Teflon trough caused the rates of the controls to rise during the day. As successive runs of didecanoyl PC-amine mixtures were completed, control rates dropped due to accumulation of amine in the enzyme trough. To minimize these effects, the enzyme compartment was washed for 15 min after each run with 0.1 N HCl, rinsed, and then washed 15 min with buffer solution. Under these conditions, the controls varied less than 5% during the day. All runs were performed in the temperature range of 22–24°C.

The apparent pK_a' values of octadecylamine and the tetracaine analog were determined from studies of compression isotherms as a function of pH for mono-

[3] Abbreviations used: didecanoyl PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; dioctanoyl PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

layers of the pure amines. The pK_a' values were also determined by direct pH titration of pure micelles of each amine and of mixed micelles of each amine (20%) with dioctanoyl PC. Micelles were formed in an homogenate of 5 μmol of lipid or amine in 5 ml of 0.1 M NaCl and 5 mM $CaCl_2$. Titrations were performed with 0.01 N NaOH using a Radiometer TTT2/SBR3 pH titrator.

RESULTS

Compression studies of mixed monolayer systems. The pure tetracaine analog formed an insoluble monolayer which was stable up to approximately 20 mN/m at pH 7.0. At basic pH values, the tetracaine analog monolayer was stable to pressures above 30 mN/m and at pH 8.5, a limiting area of 30 $Å^2$/molecule was observed. Pure octadecylamine monolayers, at pH 8.5, yielded a limiting area of 20 $Å^2$/molecule at 40 mN/m, in agreement with the values previously reported by Jarvis (12) and Patil *et al.* (13). Tetracaine analog-didecanoyl PC and octadecylamine-didecanoyl PC mixtures formed stable insoluble monolayers up to 30 mN/m at all pH values examined.

Ideal mixing in a two-component monolayer is characterized by the equation described by Gaines (14): $A_{12} = A_1N_1 + A_2N_2$, where the average area per molecule (A_{12}) is an additive function of the specific molecular areas of the two components in pure films at the same pressure (A_1 and A_2) and the mole fractions of the components in the mixed film (N_1 and N_2). Any deviation from this ideal behavior indicates nonideal mixing and component interaction. The average molecular areas for the tetracaine analog-didecanoyl PC mixed monolayers as a function of the mole fraction of the two film components are shown in Fig. 1. At both 10 and 30 mN/m, a negative deviation or condensing effect was evident. Octadecylamine-didecanoyl PC mixed monolayers exhibited ideal behavior within the range of 0–0.3 mol %, as shown in Fig. 2, since there was no deviation from the additive rule in this range.

pH titration studies. The apparent pK_a' values for octadecylamine and the tetracaine analog in pure micelles and in mixed micelles with dioctanoyl PC were determined by direct pH titration. The apparent

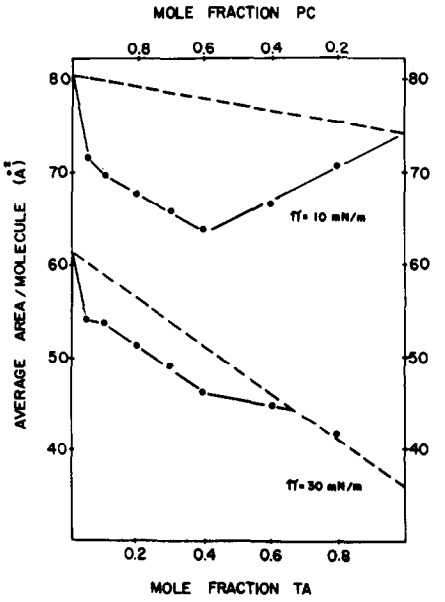

FIG. 1. Plots of average molecular area *versus* composition of didecanoyl PC-tetracaine analog (TA) mixed monolayers at π = 10 and 30 mN/m. Dotted lines represent ideal behavior calculated by the Gaines equation (14). Points connected by solid lines represent experimental data. All monolayers spread over 5 mM Tris buffer, pH 7.0, 5 mM $CaCl_2$, 0.1 M NaCl.

pK_a' for micellar octadecylamine was 8.5. In the mixed micelle, 20% mole fraction octadecylamine in dioctanoyl PC, the apparent pK_a' increased to 9.9, due to the decrease in surface pH corresponding to the decrease in micellar surface change at the same bulk pH. Similarly, the tetracaine analog had an apparent pK_a' of 6.3 in the pure micelle and 7.6 in a mixed micelle of 20% mole fraction tetracaine analog in dioctanoyl PC. Similar apparent pK_a' values were also determined from compression curve studies as a function of pH for the pure amine monolayers.

Kinetic studies. The kinetics of phospholipase A_2 action on a lecithin monolayer are characterized by a nonlinear induction phase (presteady state) followed by a linear steady state phase. The following kinetic expression for the steady state rate, as developed by Verger *et al.* (15) for phospholipase action on a monolayer, was used for analysis of kinetic data:

$$v_m = \frac{k_{cat}E_oS}{K^*_m k_d/k_p}$$

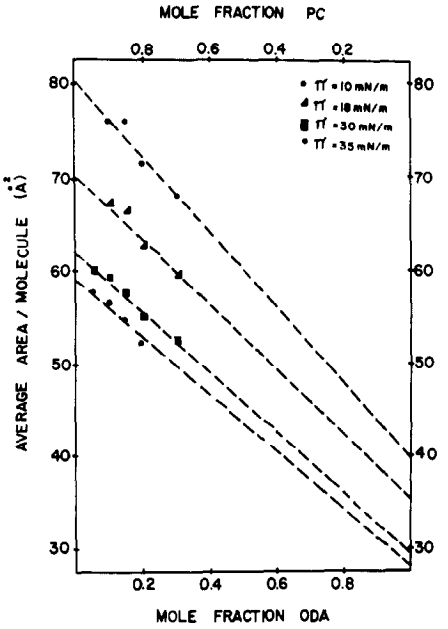

FIG. 2. Plots of average molecular area *versus* composition of didecanoyl PC-octadecylamine (ODA) mixed monolayers at various surface pressures. ●, 10 mN/m, ▶, 18 mN/m, ■, 30 mN/m, ○, 35 mN/m. Dotted lines represent ideal behavior calculated by the Gaines equation (14). All monolayers spread over 5 mM Tris buffer, pH 7.0, 5 mM $CaCl_2$, 0.1 M NaCl.

Where v_m is the velocity (molecules/surface/time); k_{cat}, the catalytic rate constant (time^{-1}); E_o, the total enzyme concentration (molecule/volume); S, the substrate concentration (molecules/surface); k_d, the desorption rate constant (time^{-1}); k_p, the penetration rate constant (volume/surface/time); and K^*_m, the interfacial Michaelis-Menton constant (molecules/surface). The reaction rate as determined with the "zero order trough" is in terms of area change (of the lipid reservoir trough)/min. This value divided by the surface area (of the reaction trough) gives the rate in terms of v_m/S (min^{-1}).[4] The rate determined in the presence of inhibitor, v_m'/S', is expressed as a relative rate, $R = (v_m'/S')/(v_m/S)$, where v_m/S is the rate of the uninhibited control with the same total enzyme concentration. The induction time, t_i, was determined by extrapolating the linear steady state rate to zero area change. The percentage of inhibition is 100–100R.

Although decanoic acid is very rapidly desorbed from neutral monolayers (16), its desorption in the presence of an insoluble amine may be slower and thus rate-limiting in the kinetic studies. To evaluate this, the following experiments were done. A mixture of didecanoyl PC:octadecylamine:decanoic acid (4:1:5 mole ratio) was spread on a pH 7 buffer. The decanoic acid rapidly desorbed, so that in the 3–4 min required for spreading the area at 10 mN/m was the same as the area occupied by didecanoyl PC:octadecylamine (4:1) alone. Decanoic acid (benzene solution) was spread on a monolayer of didecanoyl PC:octadecylamine (4:1) at 10 mN/min over pH 7 buffer. Based on the half time for the decrease in π after the addition of decanoic acid, a desorption rate constant, $k = 0.115$ s^{-1}, was calculated. This is much greater than the maximum rate measured for the phospholipase controls, $v_m/S \approx 2.7 \times 10^{-3}$ s^{-1}.

Phospholipase action on mixed PC-amine monolayers necessarily results in an increase in the mole fraction of amine in the monolayer as PC is removed. Assuming that the excess amine does not diffuse back into the reservoir trough, but remains in the enzyme trough, the change in composition of the monolayer in the enzyme trough through the course of a reaction can be calculated. In the region where the linear steady state rates were measured, the mole fraction of amine may have increased by 50%. If diffusion of amine into the larger reservoir trough occurs, then the increase in mole fraction of amine would be significantly less. Thus, the actual mole fraction of amine giving a specified percentage of inhibition may be as much as 50% greater than stated. At a high percentage of inhibition (slow rate), the change in mole fraction of amine would be much less since the amount of PC removed during the time course of the reaction is less. Kinetic data from a typical experiment with 10% octadecylamine are shown in Fig. 3. The rate of

$$^4 v_m/S = \frac{\text{rate area change-reservoir trough } (\text{cm}^2/\text{min})^*\text{S}(\text{molecules}/\text{cm}^2)}{\text{area-enzyme trough } (\text{cm}^2)^*\text{S}(\text{molecules}/\text{cm}^2)} = \text{min}^{-1}.$$

barrier movement was linear between 10–45% maximum; above that point the rate began to drop due to an accumulation of amine. The induction time determined by extrapolation of the linear portion may be underestimated due to the increasing inhibition, but is certainly less than 10 min.

Preliminary results, at pH 7.4, with the tetracaine analog- and octadecylamine-didecanoyl PC mixed monolayer systems are shown in Table I. Similar inhibition was observed with both the procine pancreatic and *C. adamanteus* enzymes on each type of mixed monolayer. Inhibition increased with increasing amine concentrations in both of the mixed films but inhibition was greater with octadecylamine than with the tetracaine analog at the same mole fraction at pH 7.4 (80% inhibition of both enzymes with 20 mol % octadecylamine as compared to 50% inhibition of both enzymes with 20 mol % tetracaine analog).

In order to determine whether the differences in inhibition by octadecylamine and the tetracaine analog were due to their different pK_a' values and thus different degrees of protonation in the mixed films at a bulk pH of 7.4, or due to other effects

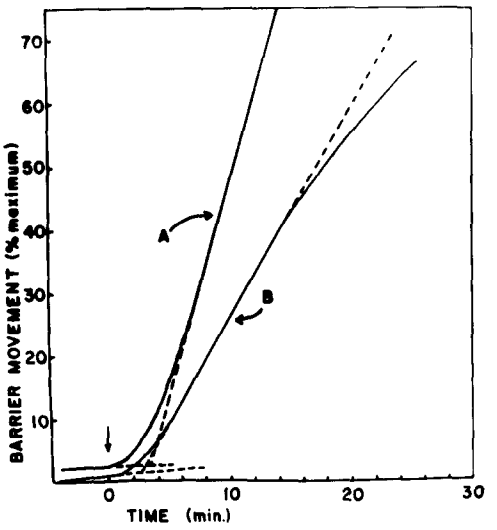

FIG. 3. Experimental kinetic data. Plot of barrier movement *versus* time. A, control-didecanoyl PC; B, 10% octadecylamine/didecanoyl PC. Maximum barrier movement = 21.7 cm; π = 10 mN/m, pH = 7.4. Pancreatic phospholipase A_2 added at zero time (arrow).

TABLE I

PERCENTAGE OF INHIBITION OF TWO PHOSPHOLIPASES A_2 ON DIDECANOYL PC-AMINE MIXED MONOLAYERS[a]

Amine Mole Fraction	Didecanoyl PC-Tetracaine Analog[b]		Didecanoyl PC-Octadecylamine[c]	
	Pancreatic PLA_2 (%)	*C. adamanteus* PLA_2 (%)	Pancreatic PLA_2 (%)	*C. adamanteus* PLA_2 (%)
0.1	29.3	28.3	41.6	42.2
0.2	47.5	54.0	83.3	79.3
0.3	67.9		100.0	
0.4	75.2			
0.5	100.0			
0.8	100.0			

[a] All monolayers spread over 5 mM Tris buffer, pH 7.4, 5 mM $CaCl_2$, 0.1 M NaCl. All rates relative to controls of pure didecanoyl PC ± 5% error in inhibition values.

[b] All didecanoyl PC-tetracaine analog monolayers spread to 7 mN/m.

[c] All didecanoyl PC-octadecylamine monolayers spread to 10 mN/m.

TABLE II

PERCENTAGE OF INHIBITION OF PANCREATIC PHOSPHOLIPASE A_2 ON DIDECANOYL PC-AMINE MIXED MONOLAYERS AT VARIOUS pH VALUES[a]

pH	Didecanoyl PC-tetracaine analog (4:1) (%)	Didecanoyl PC-octadecylamine (4:1) (%)
5.0	97.5	
6.0	77.9	94.3
7.4	47.5	83.3
8.5	0.0	71.3
9.5		51.6

[a] All monolayers were spread over 5 mM Tris-acetate buffer at acidic pH values and 5 mM Tris–chloride buffer at basic and neutral pH values with 5 mM $CaCl_2$, 0.1 M NaCl, to a surface pressure of 10 mN/m ± 5% error in inhibition values. All rates relative to controls of pure didecanoyl PC.

such as structural differences and molecular spacing, inhibition studies were completed for 20 mol % amine-didecanoyl PC films as a function of pH for the pancreatic enzyme. As shown in Table II, inhibition decreased with increasing pH and decreasing surface charge for both mixed monolayers at 10 mN/m. At low pH values (5.0–6.0) where both amines would be expected to be fully protonated, inhibition was maximal and similar on both mixed films (94 and 97%). Approximately 50% in-

hibition was observed at the pH values similar to the pK_a' values for each amine where the amines were half-protonated. At pH 8.5, where the tetracaine analog would be essentially unprotonated, no inhibition was observed. It was not feasible to study octadecylamine at pH values much above 9.5, where it would be completely deprotonated, since nonenzymatic hydrolysis of didecanoyl PC would be appreciable.

Studies of the rate of pancreatic phospholipase A_2 action as a function of surface pressure were performed on pure didecanoyl PC and on tetracaine analog (20%)-didecanoyl PC mixed monolayers (data not shown). In agreement with the results of Demel *et al.* (17), a surface pressure optimum of approximately 10 mN/m and a cutoff pressure of 18 mN/m was observed for the pure didecanoyl PC monolayer. The same surface pressure optimum and cutoff pressure was observed for the tetracaine analog-lipid mixed monolayers.

Induction times in all of the kinetic studies with didecanoyl PC-amine mixtures seemed to be similar to those with the pure didecanoyl PC monolayers.

DISCUSSION

The negative deviation exhibited by the tetracaine analog-didecanoyl PC mixed monolayers in Fig. 1 is evidence of an attractive interaction between the film components. In recent NMR studies by Yeagle *et al.* (18), restricted motion around the aromatic portion of the tetracaine molecule was observed when it was incorporated into a phosphatidylcholine bilayer, which implies that hydrophobic interactions between the two molecules are important. However, electrostatic interactions were shown to be important as well since the nmr studies also indicated that the positively charged portion of the tetracaine molecule is located at the same level in the bilayer as the negatively charged phospholipid phosphate group. The observed lack of interaction in the octadecylamine-didecanoyl PC mixed monolayers (at least up to 0.3 mol %) may be the result of structural differences between the simple amine and the anesthetic analog which result in different hydrophobic interactions with the lipid. The hydrophobic positioning of the tetracaine analog in the monolayer, involving the aromatic portion of the molecule, may enhance the attractive electrostatic interaction and result in the observed negative deviation.

Most importantly for this study however, at pH values where both amines were fully protonated (Table II), the mixed amine-didecanoyl PC monolayers yielded the same level of inhibition of phospholipase A_2 action, despite different interactions in the mixed films. This observation is evidence that inhibition by the anesthetic analog and octadecylamine is due to surface charge effects alone and not any effects related to structure or molecular spacing in the monolayer. This conclusion would be further substantiated by studies of inhibition as a function of amine concentration at a pH where the amines are both fully protonated. These data, however, have not been obtained. Surface pressure-activity studies showed that the mixed tetracaine analog-didecanoyl PC film had the same pressure optimum as did the pure lipid film, despite component interaction in the mixed monolayer. In addition, induction times with the didecanoyl PC-amine monolayers seemed to be similar to those observed with the pure didecanoyl PC monolayers. These observations tend to exclude the possibility that inhibition is due to different degrees of penetration of the pancreatic enzyme due to differences in molecular spacing in the two different films.

The conclusion that anesthetic inhibition is due to surface charge alone, and more generally, that phospholipase A_2 action is inhibited by a positively charged interface is also supported by the observation that inhibition decreases with increasing pH and decreasing surface charge; and, at values where the amines are approximately 50% protonated, only half of the maximal inhibition is observed. Thus, when the interface in the tetracaine analog-didecanoyl PC mixed monolayer is uncharged, the same rate (v_m/S) is attained as for the pure didecanoyl PC monolayer. This suggests that surface-active local anesthetics inhibit phospholipase A_2 by their surface charge

alone and not by any specific structural or spacing effects.

Hendrickson and van Dam-Mieras (5) showed that inhibition of phospholipase A_2 action by local anesthetics is not due to interference with the active site, but to enzyme-anesthetic binding which inhibits interaction between the enzyme and the interface. In those studies, long induction times (>10 min, characteristic of decreased penetration) were observed for the more surface-active local anesthetics. These anesthetics, dibucaine and tetracaine, were somewhat soluble in the subphase. The long induction times may have been due to a repulsion between the positively charged interface, created by the anesthetic molecules that had adsorbed to the surface, and the positively charged penetration site of the enzyme, created by the binding of the solubilized anesthetic monomers in the subphase. In our studies with the amines, which were totally insoluble in the subphase, similar induction times (certainly less than 10 min) seemed to be observed for the mixed films and the pure didecanoyl PC monolayers. Penetration of the phospholipase could be detected by an immediate slight rise in surface pressure after enzyme injection under both the mixed and pure lipid monolayers. It may be that the positively charged film does not necessarily inhibit penetration of the enzyme, but rather, that inhibition results from an interaction between the positively charged anesthetic analog in the interface and the penetrated enzyme. Further experiments would be necessary to substantiate this.

From the results in Table I, it is clear that both the *C. adamanteus* and porcine pancreatic phospholipase A_2 enzymes are equally affected by the positive surface charge although they have different surface pressure optima and cutoff pressures. The pancreatic enzyme has a pressure optimum around 10 mN/m and a cutoff pressure of approximately 18 mN/m, while the *C. adamanteus* enzyme has a surface pressure optimum of 12–15 mN/m and a cutoff pressure of 23 mN/m, according to Demel *et al.* (17) and our surface pressure studies. These observations support a hypothesis that inhibition may be due to anesthetic-penetrated enzyme interactions, since two phospholipase A_2 enzymes with different penetrating abilities are equally affected by the positive interface. In contrast, Wilschut *et al.* (19) showed that while porcine pancreatic phospholipase A_2 was inhibited by the presence of stearylamine in dimyristoyl PC liposomes, the activity of *Naja naja* phospholipase A_2 was unaffected by the amine.

The conclusion that surface-active local anesthetics inhibit phospholipase A_2 by their positive surface charge implies that positively charged interfaces may interfere with lipolytic enzyme action in general. Other phospholipases have been shown to be inhibited by local anesthetics (3, 4). Recently, Shier (20) showed that acyl coenzyme A:lysolecithin acyltransferase was inhibited by local anesthetics at concentrations well below normal physiological dosage and Bowley *et al.* (21) reported inhibition of phosphatidate phosphohydrolase by various amphiphilic cationic drugs. Since surface-active local anesthetics have been shown by our studies to inhibit enzyme action by charge effects rather than effects related specifically to structure, it may be that use of cationic amphipathic drugs in general have a detrimental effect on lipid metabolism. Lüllmann *et al.* (22) showed that phospholipid storage diseases are induced by several types of surface-active drugs, including local anesthetics, all of which have the similar structural feature of a hydrophobic aromatic ring with a protonated cationic side chain. Our studies suggest that phsopholipid storage diseases may result from the inhibition of phospholipase A_2 and other lipolytic enzymes due to a positively charged interface produced by the penetration of these drugs into membranes.

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