Local anesthetic inhibition of pancreatic phospholipase A_2 action on lecithin monolayers

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Abstract Using quantitative data previously reported for the penetration of local anesthetics into lecithin monolayers, the effects of surface and subphase concentrations of anesthetics on the inhibition of pancreatic phospholipase A_2 action on didecanoyl phosphatidylcholine monolayers was investigated. Inhibition as a function of subphase concentration of anesthetic was in the order: dibucaine > tetracaine > butacaine > lidocaine = procaine. Inhibition as a function of surface concentration showed no obvious correlation; procaine inhibited at a very low surface concentration, followed by lidocaine at a somewhat higher concentration, and tetracaine, butacaine and dibucaine only at rather high concentrations. Ultraviolet difference spectroscopy indicated an interaction between lidocaine and enzyme in the subphase. Fluorescence studies showed that lidocaine is a competitive inhibitor of enzyme–lipid interface interaction. It is proposed that the more surface-active anesthetics inhibit by surface effects while the less surface-active anesthetics (lidocaine and procaine) inhibit by interaction with the enzyme in the subphase, which prevents enzyme penetration at the monolayer interface.

Supplementary key words dibucaine · tetracaine · butacaine · lidocaine · procaine · ultraviolet difference spectroscopy · fluorescence spectroscopy

Several years ago Scherphof, Scarpa, and van Toorenenbergen. (1), and Waite and Sisson (2) showed that procaine-type local anesthetics inhibit phospholipase A_2 activity in liposomal, mitochondrial, and microsomal membrane systems. Recently, Künze, Bohn, and Voght (3) demonstrated local anesthetic inhibition of phospholipase A_2 activity and prostaglandin synthetase activity in homogenates of bovine seminal vesicles. These investigators concluded that local anesthetic inhibition is due to interference with calcium binding, either with the enzyme or enzyme–substrate complex, which is necessary for enzyme activity. The membrane systems used by these investigators were quite complex and subject to product accumulation. The latter effect results in a changing membrane surface during the reaction and makes kinetic analysis quite difficult. In order to avoid these difficulties, local anesthetic inhibition of porcine phospholipase A_2 was studied in a monolayer system (4, 5) with short chain lecithins where the products are completely solubilized. The quantitative study of local anesthetic penetration into lecithin monolayers presented previously (6) made possible a correlation of enzyme inhibition with surface and subphase concentrations of anesthetics. The results of this enzyme inhibition study are presented here.

EXPERIMENTAL

Materials

Porcine pancreatic phospholipase A_2 was obtained by activation of the pure zymogen by trypsin as described by Pietersen, Volwerk, and de Haas (7). Stock solutions of enzyme (1 mg/ml) for kinetic studies were prepared weekly and stored at 4°C. L-Didecanoyl PC was prepared as described by Bonsen et al. (8). Dodecyl- and hexadecyl-phosphorylcholine were prepared as described by van Dam-Mieras et al. (9). The above materials were generously supplied by Professor de Haas. Dibucaine, tetracaine, lidocaine, and procaine were supplied as the hydrochlorides by the Onderlinge Pharmaceutische Groothandel, Utrecht. Butacaine hemisulfate was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade.

Abbreviations: L-didecanoyl PC, 1,2-didecanoyl-*sn*-glycero-3-phosphorylcholine; CMC, critical micellar concentration; mN/m, milliNewtons/meter.

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Kinetic studies

Monolayer enzyme reactions were followed using the "zero order trough" as described by Verger and de Haas (4). Slow adsorption of enzyme to the Teflon trough caused the rate of the controls to rise significantly during the course of a day. To minimize this effect, fresh buffer was placed in the trough with stirring for 10–15 min between each run. Controls without inhibitor were run between each experiment with inhibitor. The rate of the controls did not vary more than 10% during the course of a day, and were quite similar before and after a particular experiment with inhibitor. The buffer for the enzyme reactions contained 0.1 M NaCl, 5 mM Tris (pH 7.0), 5 mM $CaCl_2$ (except where indicated), and varying amounts of anesthetic. The system was thermostated at 25°C. The monolayer was spread from a benzene solution of L-didecanoyl PC. The pressure was maintained at 10 mN/m, except where noted at 6 mN/m, and enzyme was added (about 250 μl of a 10 μg/ml solution) to the reaction trough with stirring. Reactions were followed for at least 20 min and up to 40 min as required for the rate to become linear.

Ultraviolet difference spectroscopy

The interactions of enzymes and lipid micelles with lidocaine were studied by UV difference spectroscopy as previously described for enzyme–substrate interaction (10). Tandem cells (2 × 1 cm) were used, with the enzyme (or lipid micelles) and anesthetic together in the sample beam and separate in the reference beam. The buffer contained 0.1 M NaCl and 50 mM Tris (pH 7.0). Titrations were carried out directly in the cells.

Fluorescence measurements

Fluorescence spectra were measured in 1 cm cells at 25°C with a Perkin-Elmer MPF-3 spectrofluorometer (Perkin-Elmer Corp., Norwalk, Conn.). The excitation and emission slit widths were set at 6 nm and the excitation wavelength was 292 nm. Titrations were carried out directly in the cells. The buffer was the same as that used for UV difference spectroscopy.

RESULTS

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, Mieras, and de Haas (5) for phospholipase action on a monolayer, was used for analysis of kinetic data:

$$v_m = \frac{k_{cat} E_0 S}{K^*_m k_d / k_p}$$

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 resevoir trough)/min. This value divided by the surface area (of the reaction trough) gives the rate in terms of v_m/S (min^{-1}). 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 percent inhibition (100–100R) at selected concentrations of various anesthetics and at different Ca^{2+} concentrations is shown in **Table 1** together with the observed induction times and the surface concentrations of anesthetics determined previously (6). The percent inhibition as a function of subphase and surface concentrations of anesthetics is shown in **Fig. 1**.

TABLE 1. Inhibition of phospholipase A_2 by local anesthetics[a]

Local Anesthetic	Conc.	t_i[b]	Inhibition	Γ[c]	Ratio LA/PC
	mM	*min*	%	*molecules/nm²*	*mole/mole*
Dibucaine	0.05	11.3	32	0.21	0.20
15 mM Ca^{+2}	0.05	14.0	39	0.21	0.20
Tetracaine	0.5	3.9	47	0.265	0.31
15 mM Ca^{+2}	0.5	4.0	47	0.265	0.31
Butacaine	0.8	4.4	23	0.29	0.33
15 mM Ca^{+2}	0.8	5.0	28	0.29	0.33
Lidocaine	1.5	4.3	27	0.078	0.08
Procaine	20.	12.8	88	0.073	0.07
	2.5	4.5	48	0.022	0.02
15 mM Ca^{+2}	2.5	6.0	48	0.022	0.02

[a] Substrate, L-didecanoyl PC, Π = 10 mN/m, T = 25°C, 0.1 M NaCl, 15 mM Tris (pH 7.0), 5 mM $CaCl_2$ (except where noted at 15 mM).
[b] Induction time. For control without inhibitor, t_i = 3–4 min.
[c] Surface concentration of local anesthetic (6).

The lack of correlation between surface concentration and inhibition for the different anesthetics, and the very low surface concentration at which procaine inhibits, suggested that inhibition might be a

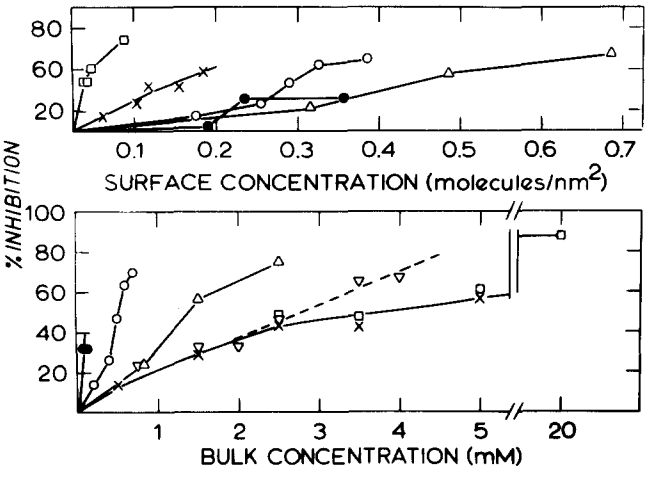

Fig. 1. Inhibition of phospholipase A_2 as a function of bulk concentration (lower) and surface concentration (upper) of anesthetic. $\Pi = 10$ mN/m: dibucaine (●), tetracaine (○), butacaine (△), lidocaine (×), procaine (□). $\Pi = 6$ mN/m: lidocaine (▽), dotted line. Conditions same as shown on Table 1.

result of interaction between anesthetic and enzyme in the subphase rather than any surface effect. The anesthetic would then reduce the effective concentration of enzyme in the subphase so that the relative rate would be the following function of inhibitor concentration: $R = K_I\ (I + K_I)$, where I is the inhibitor concentration and K_I is the dissociation constant for the inhibitor–enzyme complex in the subphase[2]. A plot of $1/R$ versus I should give a straight line through the points $1/R = 1$, $I = 0$, and $1/R = 2, I = K_I$. **Fig. 2** shows the data from Fig. 1 plotted in this way. Lidocaine and procaine show straight lines up to 5 mM, whereas the other more surface-active anesthetics show more inhibition than this model would predict (curved lines). If the inhibition by lidocaine shown by the straight line is indeed due to its interaction with the enzyme in the subphase, then alteration of its surface concentration should have no effect on this straight line. A series of kinetic studies was done with lidocaine at $\Pi = 6$ mN/m. At this lower surface pressure lidocaine penetration would be expected to be substantially increased, although no quantitative data are available for penetration at this pressure. Fig. 2 shows that at $\Pi = 6$ mN/m the same straight line fit is seen for lidocaine below 2.5 mM while above this concentration lidocaine becomes more inhibitory than the model predicts. This would indicate that, within the straight line portion, lidocaine is inhibiting by its interaction with enzyme in the subphase, with an inhibitor constant of 3–4 mM. Assuming that dibucaine, tetracaine, and butacaine interact with the enzyme in the subphase with similar inhibitor constants as lidocaine and procaine, the concentrations at which they deviate strongly from the straight lines for lidocaine and procaine would indicate the point at which inhibition by surface effects becomes important. This seems to occur at surface concentrations of 0.2–0.3 molecules/nm² and is refected by a substantial increase in the lag time (**Table 2**).

[2] $R = E_o'/E_o$, where E_o' is the concentration of free enzyme in the presence of inhibitor: $E_o' = K_I\ (EI/I)$. $E_o = E_o' + EI = EI\ (K_I/I + 1)$. $R = (K_I/I)\ (K_I/I + 1) = K_I/(K_I + I)$. The amount of enzyme at the interface is assumed to be negligible (5).

Spectroscopic studies

The UV spectrum of lidocaine consists of a large broad peak at 205 nm ($\epsilon = 16{,}300\ M^{-1}\ cm^{-1}$) and two small peaks at 271 nm ($\epsilon = 330\ M^{-1}\ cm^{-1}$) and 263 nm ($\epsilon = 420\ M^{-1}\ cm^{-1}$). The absence of absorption above 280 nm makes lidocaine suitable for the spectral studies described, in contrast to the other anesthetics that all show absorption in the 280–300 nm region. The UV difference spectrum of phospholipase A_2 and lidocaine showed a peak at 290 nm, similar to that observed for the enzyme–substrate complex (10). Measurements of ΔA_{290} as a function of lidocaine concentration, when analyzed in a double-reciprocal plot (**Fig. 3**), gave an enzyme–lidocaine dissociation constant of 10 mM ($\Delta\epsilon = 250\ M^{-1}\ cm^{-1}$).

Phospholipase A_2, when excited at 292 nm, gives a fluorescence emission peak at 343 nm (11). van Dam-Mieras et al. (9) have shown that alkylphosphorylcholine monomers (substrate analogs that bind similarly to the natural substrate, phosphatidylcholine, but are not degraded by the enzyme) inter-

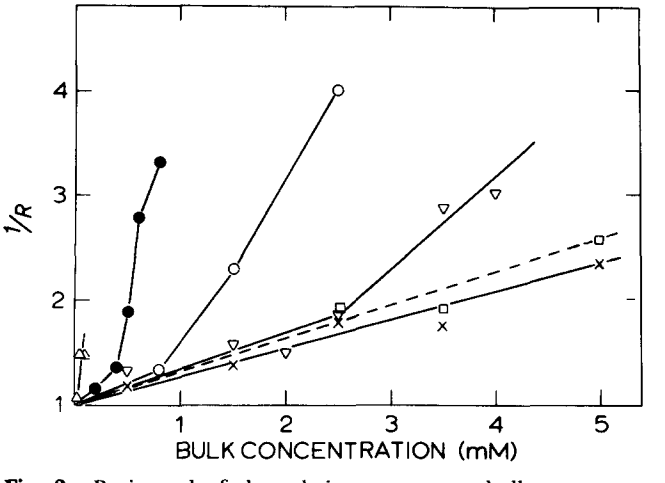

Fig. 2. Reciprocal of the relative rate versus bulk concentration of anesthetic. $\Pi = 10$ mN/m: dibucaine (△——△), tetracaine (●——●), butacaine (○——○), lidocaine (×——×), procaine (□- - -□). $\Pi = 6$ mN/m: lidocaine (▽——▽). Conditions same as shown on Table 1.

act with the enzyme, giving a small increase in quantum yield. Above the CMC, however, micellar lipids interact with the enzyme giving a large increase in quantum yield and a shift of the emission maximum to shorter wavelengths. The increase in quantum yield with monomers was interpreted as due to binding at the active site, while the changes in fluorescence with micelles were due to interaction with lipid interfaces at the so-called interfacial recognition site.

Addition of lidocaine to phospholipase A_2 produced no change in the fluorescence emission at 343 nm, when excited at 292 nm, nor did lidocaine alone give any emission under the same conditions. Titration of the enzyme with dodecylphosphorylcholine monomers below the CMC (CMC = 1.1 mM (9)) gave the same small increase in quantum yield in the absence or presence of 10 mM lidocaine, indicating no inhibition by lidocaine of substrate–analog binding to the active site. Titration of the enzyme with hexadecylphosphorylcholine above its CMC (CMC = 0.01 mM) in the presence of lidocaine, however, showed inhibition of interface interaction as evidenced by a smaller increase in quantum yield and a smaller shift to shorter wavelengths. Double-reciprocal plots of the increase in fluorescence intensity (ΔF) as a function of lipid concentration (**Fig. 4**) were characteristic of competitive inhibition by lidocaine of interface binding. The inhibitor constants calculated from these data were 5.9, 8.1, and 8.9 mM for 4.2, 8.5, and 16.6 mM concentrations of lidocaine, respectively (K_I(av) = 7.6 mM). Similar results were obtained by analyzing wavelength shifts, but these data were less accurate. Similar titrations of enzyme with micellar lipid in the presence of 5 mM $CaCl_2$ gave a similar inhibitor constant (K_I = 8.7 mM) indicating that, at least up to 5 mM there is no effect of calcium on this inhibition. With 23 mM $CaCl_2$ a somewhat larger inhibitor constant was obtained (K_I = 15 mM).

TABLE 2. Lag times as a function of surface and subphase concentrations of anesthetics

Anesthetic	Concentration	Γ	t_i[a]
	mM	*molecules/nm²*	*min*
Dibucaine	0.04	0.165	5.6
	0.05	0.21	14.
Tetracaine	0.4	0.23	4.0
	0.6	0.30	8.0
Butacaine	0.8	0.29	4.4
	1.5	0.46	7.5

[a] Induction time. Induction time for uninhibited control, 3–4 min. Π = 10 mN/m. Conditions same as shown in Table 1.

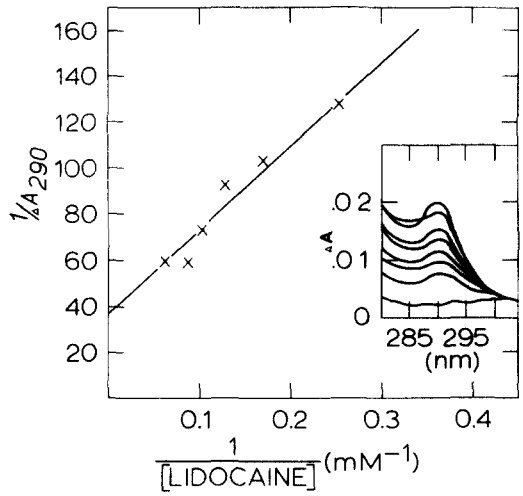

Fig. 3. Double reciprocal plot of binding data for phospholipase A_2–lidocaine obtained by UV difference spectroscopy. Experimental conditions: 106 μM phospholipase A_2, 0.1 M NaCl, 50 mM Tris (pH 7.0). **Inset**: UV difference spectra. Lidocaine concentrations (mM) (bottom to top): 0, 1.99, 3.94, 5.88, 7.80, 9.67, 11.5, 16.1.

Although the similar inhibitor constant obtained at three different concentrations of lidocaine and the fit of the data to straight lines in the reciprocal plots (Fig. 4) would indicate that lidocaine is inhibiting as a monomer and not by virtue of its incorporation into the micelles, data on the extent to which lidocaine is incorporated into the micelles was needed. This was obtained by UV difference spectral studies of the perturbation of the lidocaine spectrum by interaction with micelles. When lidocaine was titrated with hexadecylphosphorylcholine micelles, the broad absorption peak at 205 nm was observed to shift to longer wavelengths, indicative of a change to a more hydrophobic environment (12). When ΔA_{239} was analyzed with respect to lipid concentration in a double-reciprocal plot (**Fig. 5**) a dissociation constant of 60 mM was obtained. Although this is not a real dissociation constant since it is expressed in monomer rather than micelle concentration, it may be used to calculate the approximate incorporation of lidocaine into the micelle, assuming a constant number of monomers per micelle.

At 5 mM lidocaine, one can calculate a ratio of lidocaine to lipid monomer in the micelle of 0.07 for 10 mM lipid and 0.08 for 1 mM lipid[3]. These are the concentrations of lipid at the upper and lower

[3] $x = c/(K + c)$, where x is the fraction of bound lidocaine to total lidocaine, c is the concentration of hexadecylphosphorylcholine, and K is the apparent dissociation constant in terms of monomer concentration. The ratio of lidocaine to lipid monomer in the micelle equals xL/c, where L is the total lidocaine concentration.

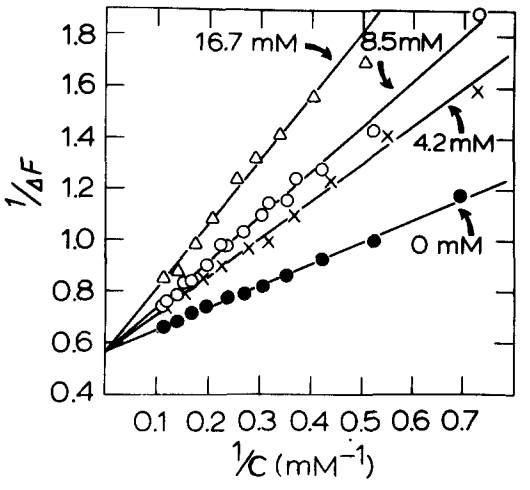

Fig. 4. Double reciprocal plot of data for phospholipase A_2–lipid micelle interaction obtained by fluorescence spectroscopy. ΔF = increase in fluorescence intensity (arbitrary units), C = concentration of hexadecylphosphorylcholine. Lidocaine concentrations: 0 mM (●), 4.2 mM (×), 8.5 mM (○), 16.7 mM (△). Experimental conditions: 0.2 mg/ml phospholipase A_2, 0.1M NaCl, 50 mM Tris (pH 7.0), T = 25°C.

ranges of the titrations shown in Fig. 4. This ratio of anesthetic to lipid in the monolayer system gave negligible inhibition of phospholipase action with the more hydrophobic anesthetics such as dibucaine and tetracaine, where high surface concentrations of anesthetic were obtained at low subphase concentrations.

Titration of 2 mM lidocaine with dodecylphosphorylcholine showed no difference spectrum up to the CMC, at which point ΔA_{239} increased sharply. The CMC determined in this way was the same (1.1 mM) as reported (9) from surface tension studies, indicating that the small incorporation of lidocaine into the micelle under these conditions does not change the micellar properties of the lipid. From these results it is concluded that inhibition by lidocaine of interface binding to the enzyme is a result of the interaction of lidocaine monomers with the enzyme.

DISCUSSION

The inhibition of phospholipase A_2 as a function of subphase concentration of anesthetic (Fig. 1a) shows the same relative potencies for the five anesthetics as reported by Scherphof et al. (1) (dibucaine > tetracaine > butacaine > lidocaine > procaine), with the exception that procaine and lidocaine inhibit to about the same degree. However, inhibition as a function of surface concentration of anesthetic (Fig. 1b) shows no obvious correlation, since procaine inhibits at a very low surface concentration and tetracaine, butacaine, and dibucaine inhibit only at rather high surface concentrations. The very low surface concentrations at which procaine inhibits suggest that inhibition may be occurring by interaction between anesthetic and enzyme in the subphase, rather than by any surface effect. The kinetic data for lidocaine and procaine are consistent with a hypothesis that these anesthetics inhibit by interaction with the enzyme in the subphase. Inhibitor constants for lidocaine and procaine from this kinetic analysis are about 3–4 mM. UV difference spectral studies with lidocaine and phospholipase A_2 indicate complex formation with a dissociation constant of about 10 mM. Fluorescence studies show that lidocaine is a competitive inhibitor of interface binding (but not monomer substrate binding) with an inhibitor constant of 7.6 mM. The similarity of these constants from the kinetic and spectral studies indicates that lidocaine interacts with the enzyme, and prevents the enzyme from penetrating the interface, the first step in the hydrolysis of interfacial phospholipids. The more surface-active anesthetics (dibucaine, tetracaine, and butacaine) might also interact with the enzyme in the subphase, although experimental evidence on this is lacking. However, since they penetrate the substrate interface more readily they may inhibit by surface effects at concentrations where possible subphase interactions might not be substantial.

To see whether there was antagonism by anesthetic of enzyme–Ca^{2+} binding, the Ca^{2+} concentration was increased to 15 mM at relatively low levels of several anesthetics in the monolayer kinetic system. Ca^{2+} at 5 mM was found to be optimal for

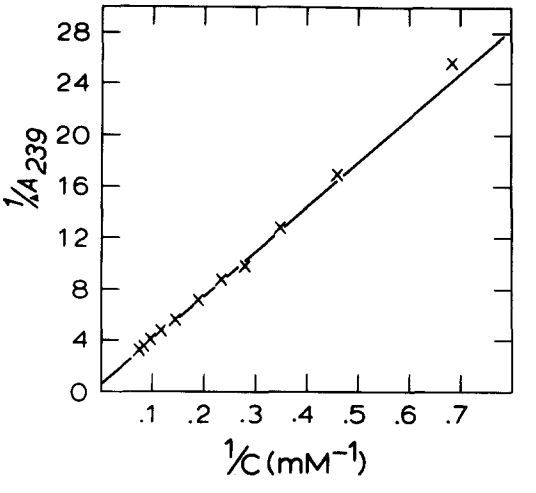

Fig. 5. Double reciprocal plot of data for lidocaine–lipid micelle interaction obtained by UV difference spectroscopy. C = concentration of hexadecylphosphorylcholine (CMC = 0.01 mM). Experimental conditions: 2 mM lidocaine, 0.1 M NaCl, 50 mM Tris (pH 7.0).

the uninhibited control, and 15 mM Ca^{2+} gave no significant increase in the control rate. The inhibition by anesthetic with 15 mM Ca^{2+} was not significantly different from that with 5 mM Ca^{2+}, nor was the induction time significantly changed (Table 1). This is in contradiction to the results of Scherphof et al. (1) and Waite and Sisson (2), and would indicate that inhibition by anesthetic is not a result of antagonism of enzyme–Ca^{2+} binding. The results of these previous investigators may be explained when one considers the effects of product accumulation in their membrane systems. Waite and Sisson (2) used phosphatidyl ethanolamine liposomes. They found that these liposomes did not bind high levels of dibucaine, although their experiments did not exclude the possibility of low level binding of anesthetic. As the phospholipase action proceeds in their system, the accumulation of fatty acids in the liposomes would increase the negative charge at the interface and greatly enhance anesthetic binding by electrostatic interaction (see discussion by Hendrickson (6)). Inhibition caused by anesthetic at the surface could then be overcome by increased Ca^{2+}, since Ca^{2+} is known to antagonize anesthetic binding to a negatively charged surface (13).

van Dam-Mieras et al. (9) recently presented evidence suggesting a second Ca^{2+} binding site on phospholipase A_2 in addition to the catalytic site. Calcium binding is required for lipid interface–enzyme interaction at alkaline pH. There is strong cooperativity between Ca^{2+} binding and interface binding, the apparent Ca^{2+} dissociation constant decreasing from about 60 mM in the absence of lipids to less than 1 mM in the presence of lipids. The effect of Ca^{2+} on lidocaine inhibition of the interface–enzyme interaction (pH 7) indicates that, up to at least 5 mM Ca^{2+}, there is no effect, but at 23 mM, Ca^{2+} may have some ability to reverse this inhibition. These data, however, are not good enough to support any conclusion, and are complicated by the strong cooperativity in Ca^{2+} and interface binding.

Scherphof and Westenberg (14) recently reported on the inhibition of pancreatic phospholipase A_2 by local anesthetics in liposomal systems of pure PC, phosphatidylethanolamine, and lipid extracts from mitochondria and microsomes. With both dibucaine and butacaine, they observed stimulation of hydrolysis at low concentrations of anesthetic followed by inhibition at higher concentrations. The stimulation at low anesthetic concentrations was ascribed to a physicochemical effect on substrate packing in the liposome. They studied the binding of $^{45}Ca^{2+}$ to the enzyme by equilibrium dialysis and found no antagonism of Ca^{2+} binding by dibucaine. They concluded that local anesthetics inhibit by interaction with the substrate rather than with the enzyme. These results are consistent with those presented here for the more surface active anesthetics. Their results, however, are complicated by the changing substrate interface as hydrolysis products accumulate. They did attempt to look at dibucaine–enzyme interactions, but their results were inconclusive, and the concentrations of dibucaine used were much lower than the concentrations of lidocaine we used to study anesthetic–enzyme interaction.

Although it is not known whether local anesthetic inhibition of phospholipase A_2 or of other lipolytic enzymes is responsible for anesthetic action or is simply a side effect, this study does suggest that interactions between anesthetic and proteins, either soluble or membrane bound, may be as important in producing anesthetic action as their effects on the membrane lipid interface. In this respect, it is interesting that Scherphof and Westenberg (15) found that delipidated membranes can take up substantial quantities of anesthetic. Of particular interest would be interactions between anesthetics and neurotransmitter receptor proteins. Finally, Strong et al. (16) recently reported that β-Bungarotoxin, a presynaptic toxin that inhibits neurotransmitter release, is a potent phospholipase A_2 and appears to act through its phospholipase activity. It would be interesting to determine what effect local anesthetics have on the activity of this neurotoxin.

H. S. Hendrickson thanks Professors van Deenen and de Haas for their generous hospitality and encouragement during the course of this work and for generous supplies of enzyme and lipids. This study was carried out during the sabbatical leave of H. S. Hendrickson from St. Olaf College, and was supported by Grant NS 11777-01 from The National Institute of Neurological Diseases and Stroke, U.S. Public Health Services.

Manuscript received 28 October 1975; accepted 5 April 1976.

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