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Action of phospholipase A_2 on bilayers. Effect of inhibitors

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Action of several solutes on the kinetics of phospholipase-A_2-catalyzed hydrolysis of the ternary codispersions containing dimyristoylphosphatidylcholine + 1-palmitoyllysophosphatidylcholine + palmitic acid is examined. The kinetics of hydrolysis is interpreted in terms of the ability of the enzyme to bind to the substrate interface. The inhibitory effect of these solutes is correlated with their ability to modify fluorescence intensity of the bound enzyme, to modify the phase-transition profile, and to inhibit aggregation/fusion of the ternary codispersions. Based on these observations, it is suggested that the solutes like *n*-alkanols, ketamine, alphadolone, alphaxalone, flufenamic acid, tobramycin, mepacrine, EMD 21657 and U-10029A modulate the phase equilibria in the codispersions and thus noncompetitively inhibit the phospholipase action. Inhibition by feverfew extract (*Tanacetum parthemium*) is also by a similar mechanism. Lipid-soluble drugs as indomethacin had little effect on the kinetics of hydrolysis. All these inhibitors decrease the total extent of hydrolysis of the available substrate. However, none of these inhibitors have any effect on the hydrolysis of monomeric substrate or on the inactivation of the phospholipase A_2 by *p*-bromophenacylbromide. These observations suggest that all these inhibitors do not interact directly with the catalytic site of the free or the bound enzyme, and their effect is primarily on the enzyme-binding sites on the substrate vesicle, that is, by perturbation of lipid-protein interaction.

Introduction

Action of phospholipase A_2 on phospholipid bilayers is regulated by their ability to bind to the substrate interface [1,2]. Binding of pig pancreatic phospholipase A_2 to bilayers apparently occurs at the organizational defect sites in the interface [3,4]. Such defects in bilayers of diacylphospholipids are for example present in unannealed vesicles [3,4] and can be induced in substrate bilayers by *n*-alkanols [5], freshly added lysophospholipids [6], and lysophospholipids + fatty acid (Refs. 7, 8 and the preceding paper). Elsewhere, it has been shown that the organizational features that enhance binding of pig pancreatic phospholipase A_2 to bilayers also stimulate aggregation/fusion of the modified vesicles [9], apparently because the defect sites act as a common locus for the fusion as well as the binding of phospholipase A_2. Several putative inhibitors for phospholipase A_2 [10] also inhibit aggregation/fusion of vesicles, presumably because they modify the defect sites in the substrate vesicles. In this paper, we have characterized the effects of several of these inhibitors on the kinetics of phospholipase-A_2-catalyzed hydrolysis and the phase properties of the ternary codispersions containing dimyristoylphosphatidylcholine + 1-palmitoyllysophosphatidylcholine + palmitic acid. The results show that the inhibition of phospholi-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pase A_2 activity results from a modification of the lipid-protein interaction mediated by the solute-induced perturbation of the phase equilibrium in the phase-separated bilayer.

Materials and Methods

All lipids used in this study were from Calbiochem or Avanti. The various inhibitors were obtained from Aldrich, Sigma and Pfaltz-Bauer or from the drug companies like Merck-Darmstadt, Parke-Davis and Adrin. Alphaxalone and alphadolone were obtained from Glaxo Laboratories, U.K. U-10029A (1-(benzylmethylamino)-3-(α,α,α-trifluoro-*m*-tolyl-oxyl)-2-propanol) was kindly provided by Dr. Donald P. Wallach of the Upjohn Company.

All the experimental protocols have been described elsewhere. The kinetics of the action of pig pancreatic phospholipase A_2 was monitored by pH-stat titrations [7,8], and the binding of the enzyme to bilayers was determined by fluorescence spectroscopy [7,8] and aggregation/fusion of the vesicles was monitored by the transmitted light at 360 nm in stirred cuvettes [9]. Typically, the reaction was initiated with 0.5 μg pig pancreatic phospholipase A_2 added to 5 ml of the reaction mixture containing the ternary codispersions at an appropriate concentration with 100 mM KCl/10 mM $CaCl_2$ at pH 8.0 and 30°C. The aqueous phase for binding and aggregation measurements also contained 20 mM Tris. Unless stated otherwise, the ternary codispersions were prepared by sonicating in a bath-type sonicator (Sonicor) a mixture of dimyristoylphosphatidylcholine/1-palmitoyllysophosphatidylcholine/palmitic acid in the mole ratio 100 : 22 : 22 ($X_p = 0.18$), or the mole ratios specified in the text. As shown elsewhere [8], this is the optimal mole ratio of substrate and product for binding and hydrolysis. Under these conditions, specific activity is about 500 I.U. Unless mentioned otherwise, the substrate concentrations refer to the concentration of dimyristoylphosphatidylcholine present as the ternary codispersions. The inhibitors, if present, were added to and equilibrated with the preformed ternary codispersions before initiating the reaction with the enzyme.

Results

Effect of n-alkanols

We have previously shown that diacylphosphatidylcholines the medium-chain *n*-alkanols ($n = 5$–12) exhibit a biphasic effect on the reaction of phospholipase A_2 with phospholipid bilayers [5]: at low concentrations, the phospholipase A_2 activity is enhanced, while at higher alkanol concentrations enzyme activity is inhibited. The maximal activity is observed at concentrations that depend upon the chainlength of the alkanol, as well as, upon the acyl chainlength and unsaturation of the substrate. We have further extended these observations. As shown in Fig. 1A, the effect of hexanol on the initial rate of hydrolysis of dimyristoylphosphatidylcholine depends upon the mole fraction (X_p) of the additives (1-palmitoyllysophosphatidylcholine and palmitic acid in 1 : 1

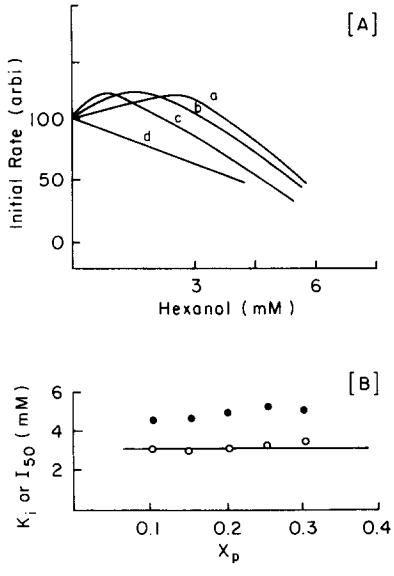

Fig. 1. (A) Effect of *n*-hexan-1-ol on the initial rate of hydrolysis of the ternary codispersions of dimyristoylphosphatidylcholine containing varying mole fractions (a, 0.1; b, 0.2; c, 0.3) of 1-palmitoyllysophosphatidylcholine + palmitic acid (1 : 1). All assays were done at 30°C, pH 8.0 in 100 mM KCl/10 mM $CaCl_2$ with 0.5 μg pig pancreatic phospholipase A_2. (B) Dependence of the 50% inhibitory concentrations I_{50} (filled circles) or K_i (open circles) of hexanol for the maximal rate of hydrolysis (obtained from the curves of the type shown in A) as a function of the mole fraction of the additives in the ternary codispersions of the substrate. K_i values were determined from the Dixon plots of the type shown in A.

mole ratio) in the ternary codispersions. The peak activating concentration of *n*-hexanol changes with increasing X_p, and the activating effect is not seen beyond $X_p = 0.20$. Thus, it appears that the activating effect of *n*-hexanol and that of lysophosphatidylcholine + fatty acid is additive at low mole fractions of these additives in the substrate vesicles.

It is particularly stricking that *n*-hexanol inhibits the initial rate of hydrolysis of the substrate in the ternary codispersions. As shown in Fig. 1B, the concentration of *n*-hexanol for 50% inhibition (I_{50}) of the initial rate of hydrolysis increases only slightly with increasing mole fraction of the products. However, as shown in Fig. 2, the I_{50} values for the homologous *n*-alkanols decreased with increasing chainlength up to decanol, and for the higher homologs the I_{50} values are higher. Like *n*-hexanol, these homologous alkanols also exhibit

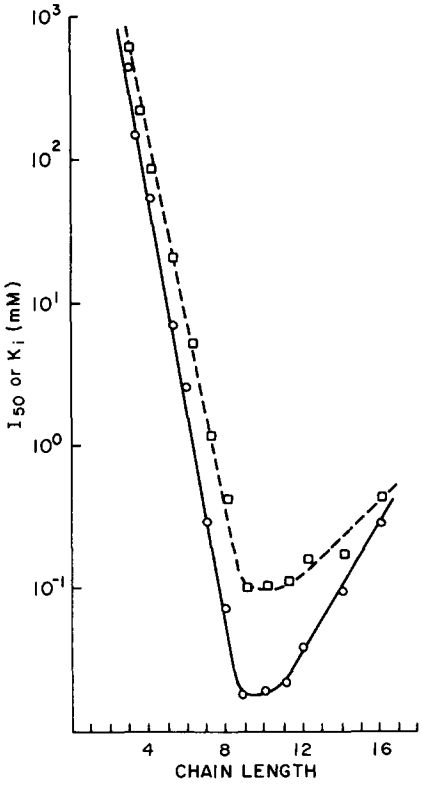

Fig. 2. Dependence of K_i (circles) or I_{50} (squares) on the chainlength of *n*-alkanol added to I_{50} the ternary codispersions. Other conditions as given in the legend to Fig. 1.

biphasic concentration dependence, and the optimal activating concentration depends upon the mole fraction of the additives. With pure diacylphosphatidylcholine vesicles, the optimal activating concentrations are 10-fold higher [4] than the I_{50} values for the ternary codispersions with $X_p > 0.1$. In all these cases, the inhibitory effect is not observed until a certain critical concentration of alkanol is present, that is, the inhibitory effect of alkanols exhibits a sigmoidal concentration dependence. Such an effect is expected if these solutes partition into the substrate bilayer, and if their effect is expressed only when a critical mole fraction of the solute is attained in the bilayer. The observations that the same alcohol can exhibit activating and inhibitory effect at the same concentration depending upon X_p, that the I_{50} values change only slightly at $X_p > 0.10$, and that the I_{50} values for the homologous alkanols decrease with the chainlength, suggest that the inhibitory effect of *n*-alkanols is due to partitioning of alkanols in the ternary codispersions rather than by a direct interaction with the enzyme.

From the K_i or I_{50} values, the incremental free energy for lower alkanols ($< C_9$) is calculated as -800 ± 20 cal/mol per CH_2 group. This suggests that, in order to exhibit their inhibitory effect, the alkanols partition into an environment whose hydrophobicity is similar to that of the interior of a bilayer [11]. In a manner analogous to the cut-off effect of higher alkanols seen for a variety of membrane effects [12,13], including the activation of phospholipase A_2 [5], the inhibitory potency of higher alkanols also decreases beyond *n*-decanol (cf. Fig. 2). Such a behavior would be expected if, for example the alkanols induced their effect by modulating the phase-equilibrium in the target bilayer.

Binding and kinetics of hydrolysis of the substrate ternary codispersions by pig pancreatic phospholipase A_2 also depends upon the phase equilibrium in the bilayer [3,4,7]. Therefore, it is of interest to examine the effect of alkanols on the kinetic parameters. The various plots (double-reciprocal, Hanes-Wolf, Dixon, Eadie-Schatchard, Cornish-Bowden) of initial rate vs. substrate ($X_p = 0.18$) concentration data [14] give $K_m = 240 \pm 30$ μM and $V_m = 500 \pm 50$ I.U. As expected, these plots are modified in the presence of *n*-hexanol.

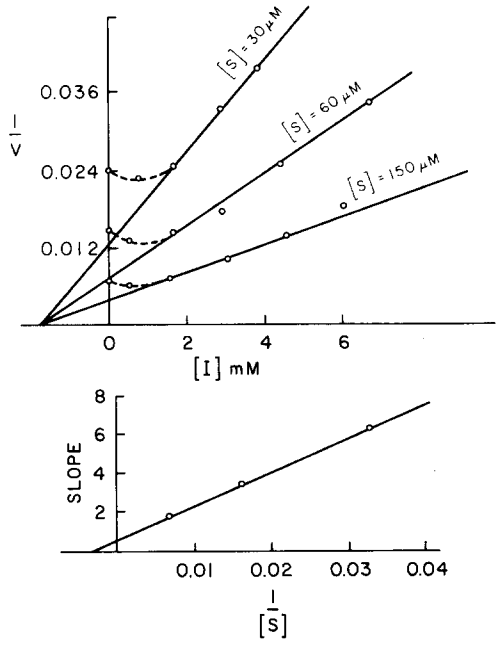

Fig. 3. Dixon plots for inhibition of hydrolysis of dimyristoylphosphatidylcholine in the ternary codispersions (X_p = 0.18) by *n*-hexanol. The kinetic parameters from such plots for other information inhibitors are summarized in Table I, Figs. 1 and 2.

Dixon plots of the initial rate in the presence of varying concentrations of *n*-hexanol (Fig. 3) show that the mechanism of inhibition is noncompetitive and $K_i = 2$ mM. As summarized in Table I, comparable values for K_m, V_m and K_i are obtained by double-reciprocal, Hanes-Wolf, Eadie-Scatchard, Dixon and Hunter-Down plots [14]. The K_i and I_{50} values for hexanol obtained at the various mole fractions of the additives are also shown in Fig. 1B, and they appear to change only slightly with X_p. The K_i values for the homologous alcohols obtained from Dixon plots are shown in Fig. 2, and all these alcohols exhibit noncompetitive type of inhibition. Noncompetitive inhibition implies a modification of the enzyme-binding site on the substrate, and a competitive inhibition would have probably meant blockage of the binding. It should be pointed out that the Dixon plots for the inhibitory effect of alkanols are linear only at higher inhibitor concentrations. As shown in Fig. 3, a departure from linearity is seen at lower inhibitor concentrations and this is quite significant for higher alkanols ($> C_8$). In this sense, the mechanistic significance of the K_i values is limited as discussed later in this paper.

Effect of U-10029A

Wallach and Brown [15] have reported that a series of 1,3-disubstituted propanes inhibit the action of venom and pancreatic phospholipase A_2. One of these derivatives, U-10029A, is found to be an effective inhibitor of the action of pig pancreatic phospholipase A_2 on the ternary codispersions. As shown in Fig. 4 and summarized in Table II, the U-10029A-induced inhibition is noncompetitive like that of alkanols, and the K_i does not change with X_p (data not shown). A striking similarity in the inhibitory effect of *n*-octanol and U-10029A is shown in Fig. 5 and in both cases, the initial rate, as well as the extent of hydrolysis are reduced in the presence of inhibitors. Addition of more enzyme at the end of the reaction progress curves does not initiate further hydrolysis, suggest-

TABLE I

KINETIC PARAMETERS (V_m, K_m, K_i) FOR THE HYDROLYSIS OF DIMYRISTOYLPHOSPHATIDYLCHOLINE IN TERNARY CODISPERSIONS (X_p = 0.18) IN THE PRESENCE OF *n*-HEXANOL (temp., 30°C)

	V_m (μmol·min^{-1}·mg^{-1})					K_m	K_i
I (mM):	0	1.5	3.0	4.5	6.0	(μM)	(mM)
Double-reciprocal	520	555	476	412	370	222	2.0
Hanes-Wolf	520	560	502	428	378	210	
Eadie-Scatchard	520	580	500	410	320	200	
Dixon						210	1.9
Hunter-Down							2.8

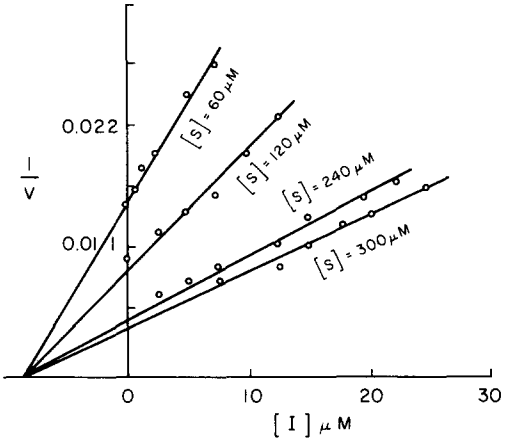

Fig. 4. Dixon plots for hydrolysis of dimyristoylphosphatidylcholine in ternary codispersions ($X_p = 0.18$) by U-10029A. The kinetic parameters are summarized in Table II.

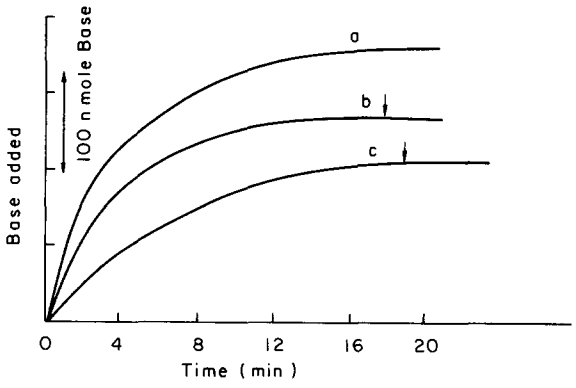

Fig. 5. Reaction progress curves for hydrolysis of ternary codispersions in the presence of (a) no additive, (b) 12 μM U-10029A and (c) 400 μM octanol. 0.5 μg phospholipase was added at time = 0 and at the points marked with the arrow. Other conditions given in the legend to Fig. 1.

ing that the excess substrate is not available to the enzyme.

Effect of other inhibitors

Several other inhibitors of phospholipase A_2 have been reported [18–20]. As summarized in Table III, these structurally dissimilar solutes inhibit the action of phospholipase A_2 on the ternary codispersions ($X_p = 0.18$). The I_{50} values for these solutes are over 1000-fold concentration range; however, there does not appear to be an obvious correlation between their lipid solubility and I_{50} values. It is particularly important to note that several solutes (e.g., ketamine, alphaxalone) activate the rate of hydrolysis (over 2-fold) at lower concentrations, whereas these same solutes are inhibitory at higher concentrations. Aqueous and enthanolic extract of feverfew plant (*Tanacetum parthenium*) also noncompetitively inhibit the phospholipase A_2 action (data not shown). All such compounds exhibit noncompetitive type of inhibition and they lower the extent of hydrolysis.

Effect of inhibitors on binding

Elsewhere, we have shown that the binding of pig pancreatic phospholipase A_2 to the ternary codispersions containing ditetradecylphosphatidylcholine is accompanied by an increase in the fluorescence intensity [7], and that there is a correlation between the binding and kinetic parameters (Ref. 8, and unpublished results.) As shown in Fig. 6, the increase in fluorescence intensity that is

TABLE II

KINETIC PARAMETERS FOR THE INITIAL RATE OF HYDROLYSIS OF THE TERNARY CODISPERSION WITH PIG PANCREATIC PHOSPHOLIPASE A_2 IN THE PRESENCE OF U-10029A

Plots	K_m (μM)					K_i at 30°C (μM)
I (μM):	0.00	1.75	3.50	5.25	8.7	
Double-reciprocal	225	300	342	402	480	14.9
Dixon	212	300	315	412	465	18.5
Hanes-Wolf	220	300	330	400	440	
Eadie-Sscatchard	215	302	330	410	473	
Hunter-Down						15.7
Average	218	300	334	408	464	16.4

TABLE III

NONCOMPETITIVE INHIBITORS

U-10029A is 1-(benzylmethylamino)-3-(α,α,α-trifluoro-*m*-tolyl-oxy)-2-propanol; EMD 21657 is a drug effective in treatment of alcoholic organic brain syndrome; it is 4-*N*-ethyl-aminoethyl-2-methyl-3-hydroxy-5-*S*-methylthiomethylpyridine [19].

Inhibitor	K_i (μM)
Methanol	$2.46 \cdot 10^6$
Tetrahydrofuran	$12.5 \cdot 10^3$
Halothane	$12.3 \cdot 10^3$
Ketamine	$4.5 \cdot 10^3$
Streptomycin sulfate	$1.6 \cdot 10^3$
Alphadolone	$1.3 \cdot 10^3$
Gentamycin sulfate	$1.3 \cdot 10^3$
Chloroform	780
Alphaxalone	779
Tobramycin	350
EMD 21657	250
n-Tetradecanol	98
Butacaine	84
Mepacrine	59
trans-n-Tetradec-9-enol	55
Flufenamic acid	50
Fluphenazine	48
Chloropromazine	27
cis-n-Tetradec-9-enol	26
U-10029A	16

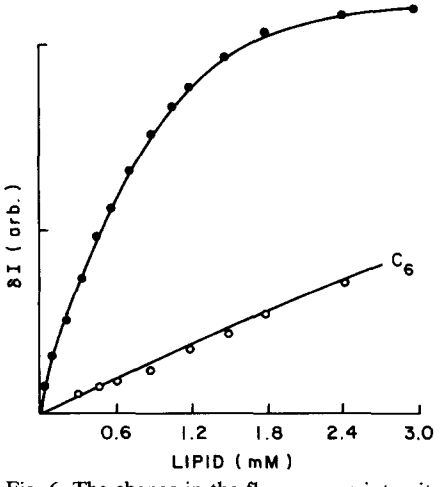

Fig. 6. The change in the fluorescence intensity at 333 nm (δI in arbitrary units) of pig pancreatic phospholipase A_2 (7 μM, pH 8.0, 30°C in 100 mM KCl/10 mM $CaCl_2$/10 mM Hepes) by ternary codispersions of ditetradecylphosphatidylcholine + 1-palmitoyllysophosphatidylcholine + palmitic acid (100 : 22 : 22 mole ratio, $X_p = 0.18$) in the absence (top) or the presence of 13 mM *n*-hexanol. Excitation 295 nm, emission 333 nm and slitwidths 4.4 nm.

accompanied by binding of the enzyme to the diether codispersions is considerably lowered in the presence of *n*-hexanol. There is no significant effect of *n*-octanol, *cis*- and *trans-n*-tetradec-9-enol, tobramycin and U-10029A on the fluorescence properties of the free enzyme. These experiments also serve as controls to assure that the effect of the inhibitor is on the bound enzyme.

At this stage, it is difficult to ascertain whether the inhibitor-induced decrease in the fluorescence intensity is due to a decrease in the quantum yield of the bound enzyme or due to the dissociation of the bound enzyme. We favor the second possibility. As shown in Fig. 7, *n*-octanol and several other inhibitors induce a concentration-dependent decrease of the fluorescence intensity of the enzyme in the presence of the ditetradecylphosphatidylcholine-containing ternary codispersions. The slopes of the plots for the various solutes are significantly different. The solutes like ketamine and alkanols exhibit a steeper concentration dependence. If all the inhibitors acted directly and stoichiometrically on the free or the bound enzyme, we would have observed a constant slope of the dose-response curves. Similarly, this difference in the slopes could not arise from a difference in the partition coefficients of the solutes. The difference in the slopes could be due to a difference in the lipid-perturbing ability of the inhibitors. These inhibitors do modify the phase-transition profile of the ternary condispersions (data not shown, however, see Ref. 9). Unfortunately, at this stage, it is not possible to bring out any meaning-

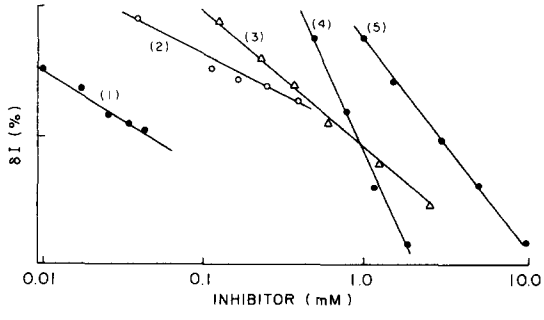

Fig. 7. Effect of inhibitors on the fluorescence intensity increase (δI) of phospholipase A_2 bound to diether ternary codispersions, $X_p = 0.18$. (1) *trans*-tetradec-9-enol, (2) gentamycin, (3) ketamine, (4) *n*-octanol, and (5) *n*-hexanol. Other conditions given in the legend to Fig. 6.

ful correlation between the phase-transition properties and the phospholipase A_2 inhibitory activity of these solutes. It may, however, be pointed out that even structurally related solutes have significantly different ability to perturb the phase equilibrium in a bilayer [5,22,23].

The concentrations for 50% decrease in the lipid-induced fluorescence change (cf. Fig. 7) are approximately the same as the K_i or I_{50} values for hydrolysis. An exact comparison is unwarranted because the lipid concentrations used for the fluorescence measurements are about 4 mM, and the inhibitors partitioned in the lipid dispersions would significantly lower the aqueous-phase concentration of the more potent inhibitors.

Elsewhere, we have shown that the sonicated ternary codispersions exhibit a change in the turbidity, indicative of aggregation/fusion [9]. Such changes in turbidity are probably due to the lateral phase separation in the ternary codispersions. Such organizational features regulate the binding and the catalytic action of phospholipase A_2 [7]. Several solutes which modify the phase equilibria in turbidity are probably due to phase separation in the ternary codispersions, the organizational feature that regulates binding and kinetic action of phospholipase A_2 [7]. Several solutes modify the phase equilibria in the ternary codispersions and thus modulate aggregation and phospholipase A_2 action. As shown in Fig. 8, the concentration for 50% inhibition of phospholipase A_2 action and for inhibition of the turbidity change exhibits a reasonable correlation for eight compounds. Similar correlation is seen for other compounds (see also Ref. 9). Such a correlation shows that the action of phospholipase and the aggregation/fusion of the ternary vesicles depend upon a common locus of bilayer organization. This locus is probably the phase boundary of laterally phase-separated domains in the bilayer, and these boundaries disappear in the presence of the inhibitors (see Ref. 9 for the calorimetric data) by shifting the phase equilibrium.

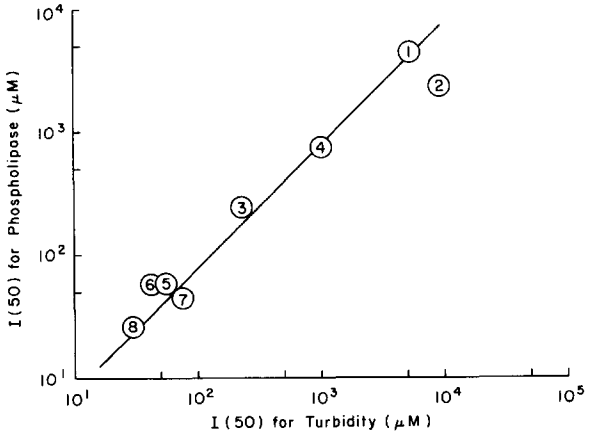

Fig. 8. Plot of the concentration of several solutes for 50% inhibition of phospholipase action (ordinate) and for 50% inhibition of aggregation/fusion as measured by turbidity change (abcissa). (1) Ketamine, (2) *n*-hexanol, (3) EMD 21657, (4) chloroform, (5) *trans*-tetradec-9-enol, (6) mepacrine, (7) chlorpromazine, and (8) *cis*-tetradec-9-enol. All measurements were made with the ternary codispersions containing dimyristoylphosphatidylcholine + 1-palmitoyllysophosphatidylholine + palmitic acid (100:22:22) at 30°C and pH 8.0.

Effect of inhibitors on the catalytic site

U-10029A, *n*-octanol and other inhibitors do not modify the fluorescence properties of phospholipase A_2 in the aqueous phase. The effect of *n*-octanol and U-10029A on the catalytic site of pig pancreatic phospholipase A_2 was examined by two different methods. 2,3-Bis(hexanoylthio)propylphosphocholine is hydrolyzed by phospholipase A_2 under monomeric substrate concentration conditions [16]. Under these conditions, the rate of hydrolysis is not modified even at 250 μM *n*-octanol or U-10029A (data not shown). Similarly, *p*-bromophenacylbromide inactivates the phospholipase A_2 by modifying the histidine residue at the catalytic site [17]. The rate constant of inactivation was not modified in the presence of *n*-octanol or U-10029A (data not shown). Such experiments demonstrate that U-10029A and *n*-octanol do not occupy the catalytic active-site region on the phospholipase A_2.

Discussion

Kinetics of action of pig pancreatic phospholipase A_2 is modulated by a variety of solutes. Most of these solutes modify the substrate interface and thereby inhibit the action of phospholipase A_2, presumably because the enzyme-interface complex or the enzyme-binding sites on the interface are modulated. The ability of solutes to modify bi-

layers and phospholipase A_2 kinetics provides an adequate criteria for identifying the mode of action of such inhibitors. The kinetic analysis of the effect of several such solutes shows that the mode of inhibition is apparently noncompetitive, that is, only V_m is altered in the presence of such inhibitors. The total extent of hydrolysis also decreases in the presence of these noncompetitive inhibitors, thereby suggesting that their effect is primarily on the substrate. While these solutes do not have any effect on the fluorescence properties of free phospholipase A_2, they lower the fluorescence quantum yield of the bound enzyme. This decrease is apparently due to a shift in the binding equilibrium because, in the presence of excess *n*-octanol, the maximal decrease in the fluorescence of the bound enzyme is almost the same as the fluorescence increase induced by the lipid.

There is considerable interest in developing a specific inhibitor of phospholipase A_2. None of the inhibitors of phospholipase A_2 reported in the literature appear to act directly on the enzyme. The protocol outlined in this paper can be used to discern the various possible modes of inhibition. The kinetic analysis developed for the action of inhibitors on soluble monomeric substrates and enzymes can only be used as a guide to elaborate the underlying mechanisms. The data presented here are obviously not extensive; however, it shows that by a suitable combination of complementary techniques it may be possible to discern the mechanism of inhibition of phospholipase A_2 at the substrate interface. The nature of inhibitor-induced modification of the substrate interface remains to be established; however, the data presented here and elsewhere [6–9] show that the inhibitory effect is not simply dependent upon the charge or lipid solubility. The fact that the extent of hydrolysis is lowered by the inhibitors suggests that the inhibitors modify the organizational defects that acts as phospholipase-A_2-binding sites. The inhibitor-induced modification of the defects is probably due to modification of the phase equilibria in the bilayer.

Acknowledgements

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