Mechanism of Cationic Amphiphilic Drug Inhibition of Purified Lysosomal Phospholipase A_1†

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ABSTRACT: Cationic amphiphilic drugs like chlorpromazine, propranolol, and chloroquine inhibit lysosomal phospholipase A in vitro. Some workers have proposed that cationic amphiphilic drugs inhibit the activity of phospholipase A_1 by forming substrate–drug complexes which cannot be degraded while others have reported competitive inhibition implying drug effects on the enzyme. To analyze the mechanism of inhibition, we examined the binding ability of these drugs to unilamellar vesicles of dioleoylphosphatidylcholine and correlated these results with a detailed kinetic analysis of phospholipase A. Chlorpromazine and propranolol bound to small unilamellar liposomes of dioleoylphosphatidylcholine substrate in a positive cooperative way consistent with two binding sites: a high-affinity site with low capacity and a low-affinity site with high capacity. The affinity of chlorpromazine for the high-affinity site was 2 times greater than that of propranolol (K_A = 13 807 ± 1722 vs. 8481 ± 1078 M^{-1}), and the saturation number for chlorpromazine was 3 times greater than for propranolol (N = 0.20 ± 0.004 vs. 0.07 ± 0.02 mol of drug/mol of phosphatidylcholine). Chloroquine did not bind to unilamellar liposomes of dioleoylphosphatidylcholine. We carried out detailed kinetic studies using purified lysosomal phospholipase A_1 from rat liver. In the case of chloroquine inhibition, the Lineweaver–Burk double-reciprocal plots showed straight lines, but the slope replots were curved, indicating the formation of complexes having 2 mol of chloroquine/mol of enzyme (EI_2 complexes). Thus, chloroquine is a competitive inhibitor which forms EI_2 complexes with phospholipase A_1. However, in the case of chlorpromazine and propranolol, the observed kinetic data do not fit to the same equilibrium used for the case of chloroquine. If the concepts of free drug, free substrate, and enzyme–substrate–drug complex (ES_bI_b) are introduced into the equilibrium developed for chloroquine, two general types of solutions can be obtained which fit to the observed data points. Values for free drug and free substrate were calculated from the results obtained from drug binding experiments carried out in parallel under conditions identical with the inhibition studies. In the first type of solution, the formation of ES_bI_b is insignificant, and in the other type of result, ES_bI_b formation occurs, but it is converted to product at a rate which is nearly identical with that of the enzyme–substrate complex.

Many cationic amphiphilic drugs have been reported to induce phospholipid storage disease in various tissues of humans and experimental animals and in cultured cells (Yamamoto et al., 1971; Lüllman et al., 1978; Lüllman-Rauch, 1979). The cellular phospholipid content increases, and it can be explained completely by the increase in the phospholipid content of lysosomes (Matsuzawa & Hostetler, 1980a). Morphological studies also show that the polar lipid accumulation appears in lysosomes (Abraham et al., 1968) and, in addition, it has been shown that several cationic amphiphilic drugs such as chloroquine accumulate in lysosomes to concentrations as high as 70 mM (Matsuzawa & Hostetler, 1980a; Hostetler et al., 1985). It has been proposed that drugs block with phospholipid catabolism in the lysosomes (Matsuzawa & Hostetler, 1980a; Hostetler et al., 1985). Redirection of phospholipid biosynthesis toward acidic phospholipids has also been proposed as a mechanism of drug-induced lipidosis (Allan & Michell, 1975; Matsuzawa & Hostetler, 1980c). Cationic amphiphilic drugs inhibit purified lysosomal phospholipase A_1 in vitro (Pappu & Hostetler, 1984; Hostetler et al., 1985), but the mechanism of inhibition is controversial. The binding of drugs to phospholipid has been proposed to be the major cause for explaining the mechanism of phospholipase inhibition by these drugs (Lüllman et al., 1978; Lüllman-Rauch, 1979). However, Kunze et al. (1982) have shown competitive inhibition of phosphatidylethanolamine catabolism using a crude lysosomal phospholipase preparation, suggesting drug–enzyme interactions.

In this report, we have examined the mechanisms of drug inhibition of phospholipase A_1 using a highly purified enzyme isolated from liver lysosomes. Three cationic amphiphilic drugs, chlorpromazine, propranolol, and chloroquine, have been used. Binding of these drugs to small unilamellar vesicles of dioleoylphosphatidylcholine has been measured, and the effects of these drugs on the ability of highly purified lysosomal phospholipase A_1 to hydrolyze small unilamellar vesicles of dioleoylphosphatidylcholine have been determined. A model for these interactions has been developed and fitted to the actual data points obtained from kinetic studies.

Materials and Methods

Preparation of Dioleoylphosphatidylcholine (DOPC) Unilamellar Vesicles. Vesicles for assays of phospholipase A and the drug binding studies were prepared as follows: 4–12 μmol of [1-^{14}C]dioleoylphosphatidylcholine (specific activity

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0.24–0.36 mCi/mmol) (enzyme assay) or unlabeled DOPC in chloroform/methanol (2/1) (binding studies) was placed in a thick-wall glass tube, and the solvent was removed by a stream of nitrogen. To the dried lipid film was added 2 mL of 2 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.4, with 20 mM NaCl), and the tube was vortexed gently for 1 min, 5–15 times with an interval of 1 min of standing on ice for the swelling. The lipid suspension was then sonicated for 20 min with the cuphorn of a Heat Systems sonicator (Model W-225R) at maximum output. The clear solution was centrifuged at 15 °C at 100000*g* for 1 h in a 50 Ti rotor (Beckman Instruments) to sediment multilamellar liposomes. Small unilamellar vesicles (SUV) remain in the supernatant fraction and were used either as a substrate or as unlabeled liposomes in the drug binding studies. The recovery of dioleoylphosphatidylcholine in the SUV fraction ranged from 81% to 86%.

Drug Binding to Small Unilamellar Vesicles of Dioleoylphosphatidylcholine. The principle of the assay for drug binding to liposomes is that the radioactive-labeled drug which binds to small unilamellar vesicles will sediment with the liposomes. Thus, the decrease of radioactive drug in the supernatant upon centrifugation represents the drug bound to liposomes. Unlabeled unilamellar liposomes of dioleoylphosphatidylcholine, 0.8 mM, and 50 mM sodium acetate buffer, pH 4.4, were used to achieve the same conditions utilized for the assay of phospholipase A, and 0.01–10 mM drug was added in a total volume of 0.2 mL. The mixtures were centrifuged in a Beckman 42.2 Ti rotor at 15 °C at 209000*g* for 16 h. Drug without added liposomes was also subjected to centrifugation as above in order to obtain control values for drug–liposome binding over the same drug concentration range as with the drug–liposome mixtures. The weight change of tubes before and after spin was also measured to adjust for evaporation during the 16-h spin.

To verify that centrifugation at 209000*g* for 16 h is sufficient to sediment the small unilamellar vesicles of dioleoylphosphatidylcholine (DOPC), radioactively labeled liposomes were prepared by using [1-^{14}C]DOPC. After centrifugation for 16 h, 94.5% of the [^{14}C]DOPC was in the bottom half and pellet while the top quarter of the supernatant contained only 0.1% of the initial amount of liposomes and the second quarter of the supernatant contained 5.4%. Therefore, to determine the amount of free drug, 50-μL aliquots were taken from the top quarter which was essentially free of liposomes. Centrifugation of drug without liposomes also caused some decrease in the amount of drug in the supernatant fraction, up to 17% of the total drug for chlorpromazine and 10% for propranolol, apparently because the drugs from micelles or aggregates which sediment. The binding data were therefore corrected for this by subtracting the decrease in supernatant drug centrifuged in the absence of liposomes. Although the weight change of tubes due to evaporation was less than 2% of the initial weight, we also corrected the data for this factor.

Purification of Liver Lysosomal Phospholipase A. Lysosomal phospholipase A_1 was purified as previously described (Hostetler et al., 1982). Eighteen male rats of Sprague-Dawley strain were killed by cervical fracture. One hundred and thirty-five grams of liver was homogenized in 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.4) and 2 mM ethylenediaminetetraacetic acid (EDTA). After sedimentation of the nuclei and cell debris fraction (by 800*g* for 5 min), the postnuclear supernatant fraction was centrifuged at 17000*g* for 15 min. The pellet (M + L, or composite lysosomal fraction) was resuspended in 10 mM potassium phosphate, pH 7.4, and subjected to three cycles of freezing and thawing. Membranous material was sedimented by centrifugation at 120000*g* for 1 h. The supernatant soluble fraction was adjusted to 20% glycerol and applied to a hydroxyapatite column (1.6 × 2.8 cm) equilibrated with 8 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol. The breakthrough fraction was applied to a concanavalin A–Sepharose column (1.6 × 5.8 cm) equilibrated with the same buffer. Phospholipase A was eluted with 0.5 M methyl α-mannoside and 10 mM EDTA in 8 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol. After concentration and rapid replacement of the buffer by 25 mM Tris–formate, pH 7.4, containing 20% glycerol using an Amicon ultrafiltration cell, the sample was subjected to chromatofocusing using a 1 × 6 cm column of polybuffer PBE exchanger (Pharmacia Fine Chemicals), equilibrated with the same buffer. The active phospholipase A fraction (pK = 5.4) was eluted by pH 4.0 polybuffer 74 (Pharmacia Fine Chemicals) (diluted with 1/8 with water/20% glycerol), collected, concentrated by ultrafiltration, washed with 5 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 20% glycerol, concentrated, and applied to a Sephadex G-150 column (2.75 × 90 cm) equilibrated with the same buffer. The fractions containing the purified phospholipase A_1 (approximate molecular weight of 34000) were adjusted to 50% glycerol and stored at −60 °C until use. The two phospholipase A_1 preparations were purified 3000–4900-fold over the homogenate, respectively.

Assay of Phospholipase A. The incubation mixture contained various amounts of [1-^{14}C]DOPC as small unilamellar vesicles in 50 mM sodium acetate, pH 4.4, in a total incubation volume of 200 μL. The reaction was started by adding 0.3–0.5 μg of purified lysosomal phospholipase A_1 protein. After incubation for 30 min at 37 °C, [^{14}C]oleic acid was extracted by the Dole extraction as modified by van den Bosch & Aarsmann (1979), and 1.0 mL of heptane phase was used for liquid scintillation counting as previously described (Hostetler et al., 1982). Under the conditions described above, the production of [^{14}C]oleic acid was linear for a minimum of 40 min with or without added inhibitor (data not shown). In these experiments, two separate enzyme preparations were used. The first preparation was used for the experiments with chloroquine. The second enzyme preparation was used for the studies with chlorpromazine and propranolol, respectively.

RESULTS

Drug Binding to SUV of DOPC Liposome. Figure 1 shows binding of chlorpromazine, propranolol, and chloroquine to 0.8 mM SUV of DOPC in 50 mM sodium acetate buffer, pH 4.4. Sixty-three percent of the chlorpromazine was bound to liposomes when the total drug concentration was 0.05 mM while 32% of the propranolol bound when the total drug concentration was 0.01 mM. When the total drug concentration was increased, the ratio of bound drug, $[D_b]$, to total drug, [D], decreased. Chloroquine did not bind to unilamellar liposomes of dioleoylphosphatidylcholine through the entire concentration range in contrast to the results with chlorpromazine and propranolol. To further analyze the binding mode, we introduced Langmuir adsorption isotherms as previously reported in the case of binding of apolipoproteins A-I and A-II to triolein particles (Kubo et al., 1982).

If this drug binding to small unilamellar vesicles of dioleoylphosphatidylcholine follows saturation kinetics, the mode of binding is expressed by

$$(N[PC] - [D_b])[D_f]/[D_b] = 1/K_A \quad (1)$$

where K_A is an association constant of drug from liposome,

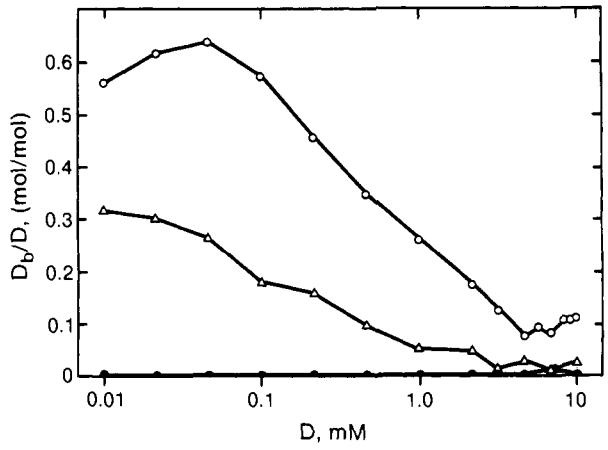

FIGURE 1: Binding of chlorpromazine, propranolol, and chloroquine to 0.8 mM SUV of DOPC in 50 mM sodium acetate, pH 4.4. Ratio of bound drug to total drug ($[D_b]/[D]$) is plotted against [D]. Data are the mean of duplicate analyses. (○) Chlorpromazine; (△) propranolol; (●) chloroquine.

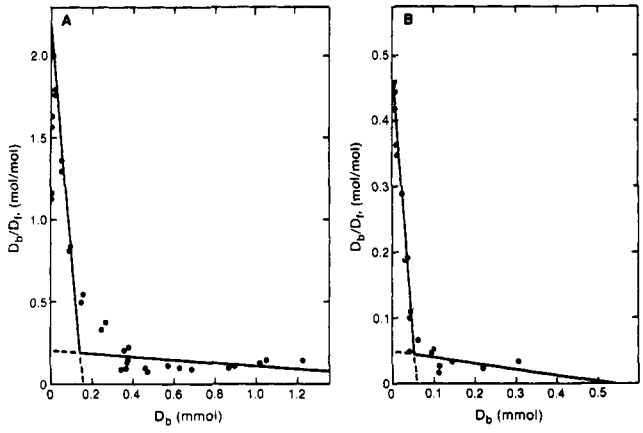

FIGURE 2: Replot of binding data of chlorpromazine and chloroquine. Each point shows the value of duplicate analyses. We regarded these hyperbolic curves as composed of two straight lines. For details of the interpretation, see the text. (A) Chlorpromazine; (B) propranolol.

[PC], $[D_b]$, and $[D_f]$ are the concentrations of dioleoylphosphatidylcholine, drug bound to liposomes, and free drug, respectively, and N is the saturation number of drug with respect to lipid in terms of moles of drug per mole of phosphatidylcholine (PC). The equation can be rearranged to

$$[D_b]/[D_f] = NK_A[PC] - K_A[D_b] \quad (2)$$

This equation gives N[PC] as an x intercept, and −(slope) gives K_A when $[D_b]$ is plotted on the x axis and $[D_b]/[D_f]$ on the x axis.

Table I: Parameters of Binding Sites of SUV of DOPC for Chlorpromazine and Propranolol

drug	binding site	K_A[a]	N[b]
chlorpromazine	app high affinity	13807 ± 1722	0.20 ± 0.004
	low affinity	98 ± 265	2.63 ± 1.02
propranolol	app high affinity	8481 ± 1078	0.07 ± 0.002
	low affinity	94 ± 187	0.67 ± 0.20

[a] Mean ± SD, units of M^{-1}. [b] Mean ± SD, units of mol of drug/mol of PC.

Table II: Kinetic Parameters for Chlorpromazine, Propranolol, and Chloroquine[a]

	app V_{max}[b]	K_s[c]	α	β	γ	K_i[c]
chlor-	22	5.6×10^{-4}	0.94	1.0	0.69	1.0×10^{-5}
promazine			0.86	0.97	0.57	1.1×10^{-5}
			34	0.52	0.69	2.5×10^{-5}
			103	0.01	0.62	2.6×10^{-5}
propranolol	21	6.4×10^{-4}	1.2	0.98	0.57	2.4×10^{-4}
			0.89	0.80	0.41	3.3×10^{-4}
			5.4	0.92	0.31	5.3×10^{-4}
			25	0.32	0.37	5.6×10^{-4}
			228	0.03	0.17	7.3×10^{-4}
chloroquine	51	8.4×10^{-4}			3.2	2.5×10^{-3}

[a] The sets of calculated kinetic parameters shown above which fit to the observed data points were obtained by the method of least squares. Initial parameter values were supplied and solutions obtained with a computer program written in Basic and used with an IBM-PC. The calculated parameters were considered to fit to the observed data points if the standard deviation of the differences between observed and calculated values of v/V_{max} was less than 0.02. The correlation coefficient (r) of the fitted parameters obtained was greater than 0.995 in each case. [b] In units of micromoles per hour per milligram. [c] In units of molar.

Replots of binding data for chlorpromazine and propranolol (Figure 2) were hyperbolic, indicating that the binding occurs in a cooperative manner. We resolved this curve into two straight lines, one representing a high-affinity binding site with low capacity and the other a low-affinity binding site with high capacity (Table I). When the two drugs are compared with regard to high-affinity binding sites, the affinity of chlorpromazine was almost twice that of propranolol (K_A = 13807 ± 1722 vs. 8481 ± 1078 M^{-1}), and its saturation number was 3 times greater (N = 0.20 ± 0.004 vs. 0.07 ± 0.02 mol of drug/mol of PC). The K_A of the low-affinity binding site was much less than that of the high-affinity one (a few percent), and for the low-affinity site, N was higher than that of the high-affinity sites in both cases.

Kinetic Data. Figure 3 shows the substrate–velocity curve of phospholipase A_1 with chlorpromazine, propranolol, and chloroquine. The symbols in Figure 3 represent the observed data points while the solid lines are calculated values obtained by using eq 7 and the kinetic parameters shown in Table II.

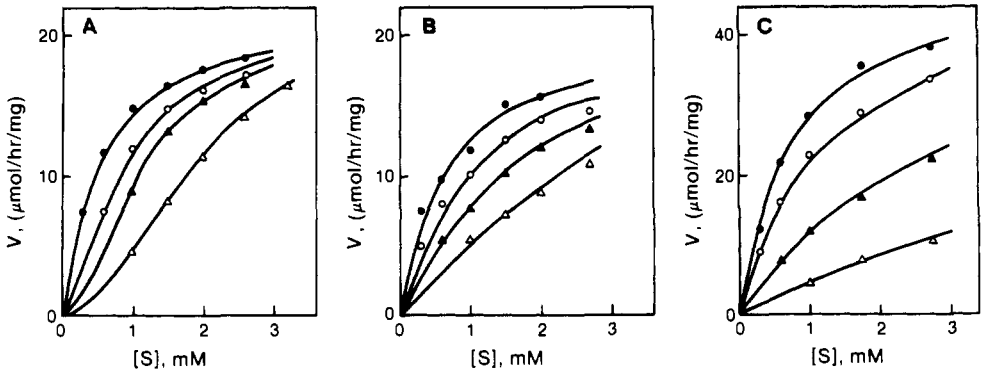

FIGURE 3: Substrate–velocity curves of kinetic data of chlorpromazine, propranolol, and chloroquine: (A) chlorpromazine; (B) propranolol; (C) chloroquine. Solid lines are calculated lines on the basis of parameters listed in Table II. (A) (●) 0, (○) 0.03, (▲) 0.05, and (△) 0.1 mM chlorpromazine. (B) (●) 0, (○) 0.08, (▲) 0.15, and (△) 0.25 mM propranolol. (C) (●) 0, (○) 1, (▲) 4, and (▲) 10 mM chloroquine.

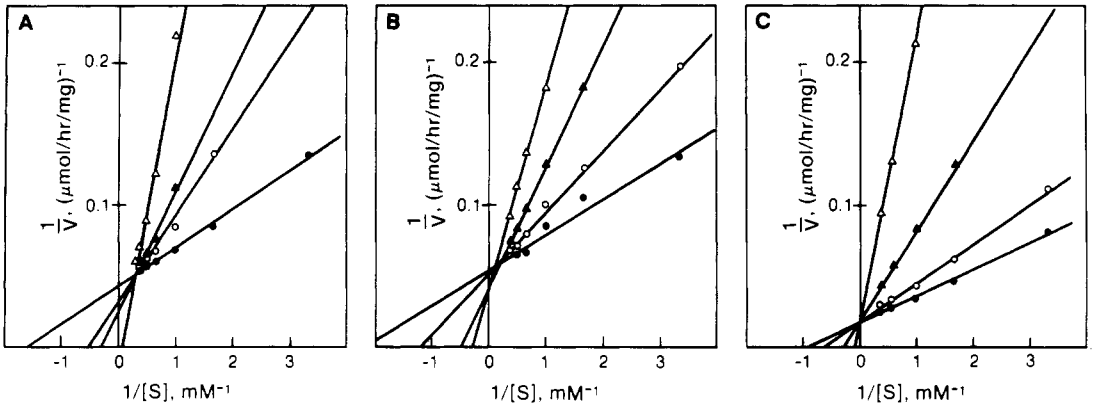

FIGURE 4: Lineweaver–Burk double-reciprocal plots of kinetic data of chlorpromazine, propranolol, and chloroquine: (A) chlorpromazine; (B) propranolol; (C) chloroquine. Symbols same as in Figure 3. The solid lines are simple linear least-squares analyses of data. In cases of chlorpromazine and propranolol (panels A and B), these lines do not solve the mechanism of inhibition.

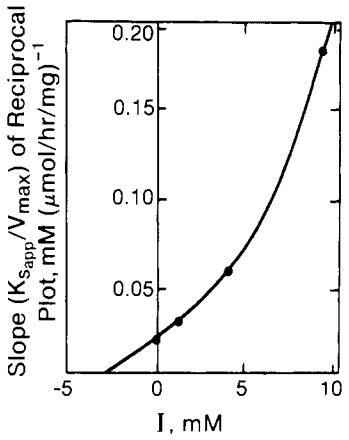

FIGURE 5: Slope replots of Lineweaver–Burk double-reciprocal plot data of chloroquine.

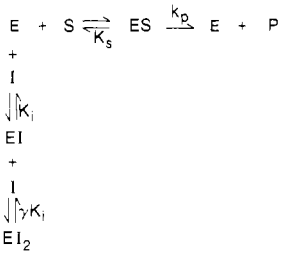

FIGURE 6: Equilibrium 1 for chloroquine.

Chlorpromazine shows a typical sigmoidal curve, while propranolol and chloroquine do not. Figure 4 shows the Lineweaver–Burk double-reciprocal plot of kinetic data with these drugs. When no drug was present, the points on each panel showed a straight line. This showed that small unilamellar vesicles of DOPC were hydrolyzed by purified lysosomal phospholipase A following the equation of Michaelis–Menten. The apparent K_m was 0.68 ± 0.15 mM. The results for chloroquine were obtained with a different enzyme preparation than that used for chlorpromazine and propranolol, and since the degree of purification of the preparations was not the same, the observed V_{max} values differ (51 and 21 μmol h^{-1} mg^{-1}, respectively).

When drugs were present, only chloroquine showed straight lines having a single intercept with the $1/v$ axis. For further analysis of the inhibition by chloroquine, slope replots ($K_{s_{app}}/V_{max}$) of the double-reciprocal plot data were used (Figure 5). This replot showed a curved line, not a straight line as expected. This indicates that phospholipase A_1 must have two binding sites for chloroquine which are not mutually

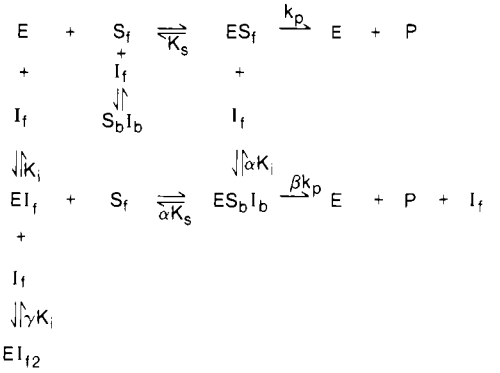

FIGURE 7: Equilibrium 2 for chlorpromazine and propranolol.

exclusive so that EI_2 can form. The equilibrium for chloroquine is shown on Figure 6. In this equilibrium (Figure 6), as well as that developed for the other drugs (Figure 7), we have taken $1/K_s$ to be the overall association constant representing the binding of the enzyme to the surface of the substrate bilayer vesicle as well as to a molecule of dioleoylphosphatidylcholine substrate. The final velocity equation is given by

$$\frac{v}{V_{max}} = \frac{[S]}{K_s(1 + [I]/K_i + [I]^2/\gamma K_i^2) + [S]} \quad (3)$$

This equation can be modified to

$$v/V_{max} = [S]/(K_{s_{app}} + [S]) \quad (4)$$

where

$$K_{s_{app}} = K_s(1 + [I]/K_i + [I]^2/\gamma K_i^2) \quad (5)$$

or

$$\frac{K_{s_{app}}}{V_{max}} = \frac{K_s}{V_{max}} + \frac{K_s}{K_i V_{max}}[I] + \frac{K_s}{\gamma K_i^2 V_{max}}[I]^2 \quad (6)$$

By applying the replots data listed in Figure 5 to these equations, we can obtain 51 μmol h^{-1} mg^{-1} as the apparent V_{max}, 0.84×10^{-3} M as K_s, 2.5×10^{-3} M as K_i, as 3.2 as γ (Table II). This equilibrium fits to our observed data and indicates that lysosomal phospholipase A_1 is inhibited by chloroquine with a competitive type of inhibition which allows two molecules of chloroquine to bind to the enzyme at the same time.

On the contrary, double-reciprocal plots of chlorpromazine and propranolol could not be analyzed in the manner used with chloroquine since these plots gave curved lines. Even when we took them as straight lines, it was not possible to determine

the mechanism of inhibition. Therefore, we introduced the concept of drug binding to substrate with the hypothesis that only the drug which is not bound to the liposome ($[I_f]$ represents free inhibitor concentration) can interact with phospholipase A_1 protein. The equilibrium we introduced is shown in Figure 7. In this case, the substrate free from drug is given as S_f, substrate bound with drug as S_b, drug free from substrate as I_f, and drug bound to substrate as I_b. The final velocity equation is

$$\frac{v}{V_{max}} = \{[S_f] + (\beta/\alpha K_i)[S_f][I_f]\}/ \{K_s + [S_f] + (K_s/K_i)[I_f] + (K_s/\gamma K_i^2)[I_f]^2 + (1/\alpha K_i) \times [S_f][I_f]\} \quad (7)$$

where $[I_f]$ and $[S_f]$ can be calculated from the binding data by using the equations:

$$[I_f] = [D_f] = [D] - [D_b] \quad (8)$$

$$[D_b] = (1/2K_A)\left(K_A N[PC] + K_A[D] + 1 - \sqrt{(K_A N[PC] + K_A[D] + 1)^2 - 4K_A^2 N[PC][D]} \right) \quad (9)$$

$$[S_f] = [PC] - [S_b] \quad (10)$$

$$[S_b] = [PC]\frac{[D_b]/[PC]}{N} \quad (11)$$

We used the numbers of the apparent high-affinity binding site for obtaining $[I_f]$ and $[S_f]$ because the contribution of the low-affinity binding site is already contained in numbers of the "apparent" high-affinity binding site at the concentrations used for studies of inhibition.

In eq 7, we regarded v/V_{max}, $[S_f]$, and $[I_f]$ as variables and K_s, K_i, α, β, and γ as constants and applied the least-squares method to determine these constants. The kinetic parameters obtained are shown in Table II. Many different sets of kinetic constants can be calculated from the observed data. All of the parameters shown in Table II fit to observed data by using eq 7. The solid lines in Figure 3, panels A and B, were generated from these calculations while the data points represent the observed kinetic values. When we did not introduce the EI_{f2} complex, fitting to the observed data points was not good, indicating that it is also necessary to have IE_{f2} complexes to explain the data obtained with chlorpromazine and propranolol.

Discussion

Our experiments demonstrate that chloroquine inhibits highly purified lysosomal phospholipase A_1 of rat liver in vitro with small unilamellar vesicles of DOPC as a substrate even though the drug does not bind to the substrate liposome. The mode of action of chloroquine on phospholipase A is competitive inhibition with formation of complexes consisting of 2 mol of drug/mol of enzyme. Thus, drug binding to substrate is not essential for inhibition as has been proposed by others (Lüllman et al., 1978). Kunze et al. (1982) reported that rat liver phospholipase A_1 activity against phosphatidylethanolamine is inhibited by chloroquine with a mode of competitive inhibition. We reexamined their data by making a slope replot of the original Lineweaver–Burk plots. The resulting slope replot was curved like the data presented in Figure 5. Thus, inhibition of liver phospholipase A_1 by chloroquine is competitive inhibition with formation of EI_2 complexes when sonicated preparations of either phosphatidylcholine or phosphatidylethanolamine are used as substrate.

Chlorpromazine and propranolol bind to DOPC in a positive cooperative manner which is consistent with two binding sites, one having a high affinity and a low capacity and the other having a low affinity and a high capacity. Chlorpromazine binds to unilammelar vesicles of DOPC with greater affinity and 3-fold higher capacity than that of propranolol as far as the high-affinity binding site is concerned. Similarly, Bickel & Steele (1974) demonstrated two types of binding sites on rat liver subcellular membrane fractions for chlorpromazine and imipramine. Lüllmann & Wehling (1979) also demonstrated two binding sites for chlorphentermine on phosphatidylserine liposomes. They proposed that hydrophobic forces are mainly responsible for the binding to phosphatidylcholine. Francesco & Bickel (1977) demonstrated two kinds of binding sites for chlorpromazine using sonicated dispersions of egg lecithin. Their affinity and capacity constants are fairly close to our results even though their method (equilibrium dialysis) and conditions, including pH, were different. Schwendener & Weder (1978) reported a two-phase process for binding of chlorpromazine to single-bilayer liposomes of egg yolk lecithin. They proposed that one occurs at the liposome surface in a fast process and the other occurs at a lipophilic phase in a slow process. Our data are obtained at steady state but could be consistent with these findings if the high-affinity binding site is the liposome surface and the low-affinity site is in the interior of the lipid bilayer. Lüllman & Wehling (1979) reported that propranolol bound to egg yolk phosphatidylcholine liposomes at pH 7.4 but chloroquine did not bind. Although our studies were done with DOPC at pH 4.4, the present results are in general agreement with the prior studies.

Kinetic studies of chlorpromazine and propranolol inhibition of purified phospholipase A_1 can be analyzed by equilibrium 2 (Figure 7), which has the concept that the drug which is not bound to liposomes (I_f) functions as an enzyme inhibitor (I_f) and that ES_bI_b complexes are formed and hydrolyzed. We obtained several sets of kinetic constants by computer analysis which fit to the observed experimental data points, and the results are shown in Table II. In these sets of data having high values of α such as 25, 34, and so on, the presence of the ES_bI_b complex itself is negligible. Therefore, the value of β has no meaning in these circumstances. In these cases, equilibrium 2 is essentially identical with equilibrium 1 (Figure 6) except that the amount of drug which interacts with enzyme protein (EI_f) decreases because of binding of drug to substrate (S_bI_b). In cases where low values of α were found, such as 0.94, 0.86 (chlorpromazine) and 1.2, 0.89 (propranolol), the presence of ES_bI_b complex is significant. Therefore, it is useful to examine the value of β, which represents the rate of conversion of ES_bI_b to product. As shown in Table II, when α is small, β ranges from 0.8 to 1.0 times the hydrolytic rate of ES_f. In other words, substrate bound with drug (ES_bI_b) can be hydrolyzed with an identical or only slightly lower velocity than ES_f. This represents partial competitive inhibition with formation of EI_{f2} complexes with reduction of the inhibitor concentration ($[I_f]$) because of inhibitor binding to substrate. We cannot determine which sets of kinetic parameters fitting to the data points describe the actual experimental situation because we have no way to determine the amount of ES_bI_b complex which is present.

Robinson & Waite (1983) reported that the introduction of a positive surface charge to DOPC vesicles by the addition of stearylamine does not cause a significant change in the hydrolysis rates with a purified lysosomal phospholipase A_1 from rat liver. This observation coincides with our present observation that the introduction of positive charge on the DOPC substrate by chlorpromazine and propranolol does not alter the rate of conversion of ES_bI_b to product (β is 0.8–1.0 when α is small).

In summary, with cationic amphiphilic drugs which can bind to substrate liposomes, such as chlorpromazine and propranolol, it has been postulated that the binding of drugs to the phospholipid substrate is responsible for inhibitory activity against liver phospholipase A_1. However, we can see that this is not the case because kinetic parameters shown in Table II indicate that the presence of the ES_bI_b complex itself is negligible when the hydrolytic rate of the ES_bI_b complex is small. Furthermore, in cases where ES_bI_b is significant, its conversion to product is almost as rapid as that of ES_f itself. We conclude that chlorpromazine, propranolol, and chloroquine inhibit liver lysosomal phospholipase A_1 by interacting with the enzyme protein. Binding of drugs to substrate decreases the amount of free drug which results in modulation of velocity–substrate curves.

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Complementary Substrate Specificities of Class I and Class II Collagenases from *Clostridium histolyticum*†

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ABSTRACT: The substrate specificities of three class I (β, γ, and η) and three class II (δ, ϵ, and ζ) collagenases from *Clostridium histolyticum* have been investigated by quantitating the k_{cat}/K_M values for the hydrolysis of 53 synthetic peptides with collagen-like sequences covering the P_3 through P_3' subsites of the substrate. For both classes of collagenases, there is a strong preference for Gly in subsites P_1' and P_3. All six enzymes also prefer substrates that contain Pro and Ala in subsites P_2 and P_2' and Hyp, Ala, or Arg in subsite P_3'. This agrees well with the occupancies of these sites by these residues in type I collagen. However, peptides with Glu in subsites P_2 or P_2' are not good substrates, even though Glu occurs frequently in these positions in collagen. Conversely, all six enzymes prefer aromatic amino acids in subsite P_1, even though such residues do not occur in this position in type I collagen. In general, the class II enzymes have a broader specificity than the class I enzymes. However, they are much less active toward sequences containing Hyp in subsites P_1 and P_3'. Thus, the two classes of collagenases have similar but complementary sequence specificities. This accounts for the ability of the two classes of enzymes to synergistically digest collagen.

Collagenases are unique in their ability to efficiently hydrolyze the triple-helical region of collagen under physiological conditions (Seifter & Harper, 1971). The resistance of collagen to digestion by most proteinases is partly due to its triple-helical structure, but is also a consequence of the primary structure of the constituent chains that have a high content of Gly, Pro, and Hyp. In order to detail the mode of attack of collagenases on native, triple-helical collagens, it is first necessary to know the sequence specificities of these enzymes. With this knowledge in hand, the influence of the secondary structure of the substrate in determining the sites and rates of hydrolysis can be addressed.

The bacterium *Clostridium histolyticum* produces two classes (designated I and II) of collagenases[1] (EC 3.4.24.3)

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