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Persistence of Complexed Acidic Phospholipids in Rapidly Mineralizing Tissues is Due to Affinity for Mineral and Resistance to Hydrolytic Attack: *In Vitro* Data

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Abstract. Acidic phospholipids, complexed with calcium and inorganic phosphate, are components of extracellular matrix vesicles. Both the complexed acidic phospholipids and matrix vesicles have previously been shown to serve as hydroxyapatite (HA) nucleators in solution and when implanted in a muscle pouch. The present study supplies evidence that complexed acidic phospholipids can persist in mineralizing tissues both because of their affinity for HA and because of their resistance to hydrolysis by phospholipase A_2. Calcium-phosphatidylserine-phosphate complex (CPLX-PS) synthesized with ^{14}C-labeled phosphatidylserine (PS) was used to measure CPLX-PS affinity for HA using a Langmuir adsorption isotherm model. The affinity was shown to be higher and more specific than that of PS itself (K = 8.66 ml/μmol; N, the number of binding sites = 20.4 μmol/m^2 as compared with previously reported values for PS of K = 3.33 ml/μmol, and N = 4.87 μmol/m^2). Incorporated into synthetic liposomes and incubated in a calcium phosphate solution in which mineralization is induced by an ionophore, CPLX-PS showed behavior distinct from free PS. As previously reported, PS in these liposomes totally blocked HA formation. On the other hand, CPLX-PS in similar concentrations had a varied response, having no effect, slightly inhibiting, or actually promoting HA formation. CPLX-PS was also shown to be a poorer substrate for phospholipase A_2 than PS, with Km = 4.63 mM for CPLX-PS and Km = 0.27 mM for PS; and V_{max} = 0.029 ml/minute for CPLX-PS and V_{max} = 0.066 ml/minute for PS. These data explain how complexed acidic phospholipids may persist in the growth plate and facilitate initial mineral deposition.

Key words: Hydroxyapatite — Complexed acidic phospholipids — Liposomes — Phospholipase A_2.

Acidic phospholipids, complexed with calcium and inorganic phosphate (Pi) [1–2] or with calcifiable proteolipids, calcium, and Pi [3–5] cause hydroxyapatite (HA) deposition *in vitro* [6–9]. HA deposition similarly occurs when these molecules are implanted *in vivo* in a muscle pouch [10]. Analyses of tissue lipid composition have shown that the concentration of these complexed acidic phospholipids (CPLX) increases where mineralization is commencing, i.e., in the lower half of the epiphyseal growth plate [11], and during initial stages of bone induction [12]. The concentration of CPLX is greater in newly mineralizing tissues than in fully mineralized tissues [13–16].

CPLX is part of the ''nucleational core'' [9] that facilitates initial mineral deposition in extracellular matrix vesicles (ECMVs). ECMVs are believed to be the site of initial mineralization in many types of physiologic mineral deposition [17, 18]. The concentration of CPLX is greater in ECMV membranes than in chondrocyte cell membranes from which the ECMVs are derived [19]. These observations suggest that CPLX is formed to prepare tissues for mineralization. However, the hypertrophic zone of the epiphyseal growth plate where mineral deposition commences is also enriched in phospholipase A_2 [20], an enzyme that hydrolyses acidic phosphatides, converting them to their lyso-derivatives. CPLX made from lyso-phosphatides do not promote *in vitro* mineralization [21], and few lyso phosphatides are found in the rapidly mineralizing tissues [14]. These data suggest that either CPLX is resistant to attack by phospholipase A_2, or that CPLX is protected by its association with the mineral phase.

The purpose of this investigation was to examine the affinity of CPLX for HA mineral and to determine whether CPLX is resistant to attack by phospholipase A_2. In addition, because acidic phospholipids incorporated into liposome models of ECMVs had been shown to retard mineral deposition [22–25], the effect of CPLX on liposome-mediated mineralization was also examined.

Materials and Methods

The lipids used in this study were obtained from Avanti Polar Lipids Inc (Birmingham, AL). Phospholipase A_2 Naja mocambique, the ionophore A23187, and Sephadex were from Sigma Chemicals (Saint Louis, MO). L-3-phosphatidyl (3-^{14}C) serine 1,2 diolein Phosphatidylserine (specific activity of 30 μC/nmol) was obtained from Amersham (Arlington Heights, IL). Fluorescein (5,6-carboxyfluorescein) was obtained from Kodak (Rochester, NY). All other reagents were obtained from Fisher (Springfield, NJ). Synthetic HA, prepared as previously described [26], had a specific surface area of 54 m^2/g.

CPLX Synthesis and Characterization

CPLX with PS as the sole acidic phospholipid component, hereafter referred to as CPLX-PS, was prepared by a modification of a

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previously described method [2]. In brief, phosphatidylserine (25 mg) dried under N_2 was dispersed in 100 ml of a calcium phosphate solution consisting of 2 mM $CaCl_2$, 1.7 mM NaH_2PO_4, and 150 mM pH 7.4 Tris (Tris-hydroxymethylamino methane-HCl) buffer. The mixture was shaken at room temperature for 8 hours, and the lipid constituents were extracted into chloroform:methanol (2:1, v/v) by repeated washings. The lower chloroform:methanol phase was dried under N_2, and any noncomplexed PS was removed by extraction with ethanol:ethyl ether (3:1, v/v) in which CPLX is insoluble. The dried lipids were then washed briefly with pH 5 water to remove nonlipid-associated calcium phosphate, resuspended in a small volume of chloroform:methanol (2:1), and stored at −7°.

Steps in the preparation of CPLX-PS were followed with labeled PS. During the synthesis, aliquots of each preparation and wash were mixed with scintillant and the % of label in each isolate was determined.

The synthetic CPLX was analyzed chemically for calcium [27], inorganic phosphate [28], and organic phosphate [29]. Alternatively, inorganic phosphate was estimated in some CPLX-PS after dissociating the CPLX-PS in 88% formic acid, and partitioning the organic and inorganic phosphate components between chloroform: methanol (2:1) and 1 N HCl, respectively [30]. Organic phosphate was also determined in CPLX-PS and liposomes by high performance TLC (HPTLC) using a developer [31] consisting of chloroform: methanol: acetic acid:H_2O (25: 15: 4:2, v/v), and Touchstone visualization [32]. The density of the spots was measured using a Helena Labs Quickscan R&D Densitometer (Beaumont, Texas) linked to a personal computer.

Table 1. Properties and composition of synthetic CPLX-PS

1. Composition of typical synthetic CPLX-PS

	mole %		
Code	Ca	P_{org}	Pi
M-3	37.8	20.2	41.8
P-0	46.9	23.9	29.1
P-2	45.2	23.8	30.4
I-0	27.7	27.7	40.0
E-1	44.1	32.1	23.7
mean ± SD	42 ± 7	26 ± 5	33 ± 8

2. Distribution of ^{14}C-PS during CPLX-PS synthesis and isolation
   Starting material PS (2×10^6 cpm) in calcium phosphate buffer
   Organic extract (1.9×10^6 cpm)
   Aqueous residue (128×10^3 cpm)
   Noncomplexed PS in ethanol:ether extract (156×10^3 cpm)
   Aqueous wash (20×10^3 cpm)
   Isolated CPLX-PS (1.42×10^6 cpm)

3. Binding affinities of CPLX-PS for HA
   K 866 ml/μmole
   N 20.4 μmole/m^2

K = affinity constant; N = number of binding sites

Adsorption Isotherm

To estimate the degree of binding between HA and CPLX-PS, synthetic CPLX-PS containing ^{14}C-labeled PS was dispersed in 150 mM NaCl, buffered at pH 7.4, and serial dilutions were used to prepare a range of CPLX-PS concentrations. Aliquots of 1.25 ml were incubated with constant shaking with 0, 1.25, 2.5, 5, or 10 mg HA for 24 hours at 37° in micro-fuge tubes. Samples at each HA concentration were studied in triplicate. An aliquot of the original solution was used to determine the specific activity of the PS in terms of cpm/μM of phospholipid. Following incubation, the nonbound CPLX-PS was separated from the adsorbed material by centrifugation in a micro-fuge (12,000 rpm). The radioactivity of the supernate was measured, and the concentration of bound CPLX-PS, calculated using a Langmuir adsorption model [33], is described by the equation:

$$C_e/Q = 1/NK + C_e/N$$

where Q is the μmoles of phospholipid bound per m^2 hydroxyapatite, calculated knowing the specific surface of the apatite. C_e is the equilibrium concentration of phospholipid in μmol Porg/ml. From a plot of C_e/Q vs C_e, an affinity constant K in ml/μmol and the number of binding sites, N, in μmol/m^2 was calculated as described previously for PS binding [26].

Seeded HA Growth

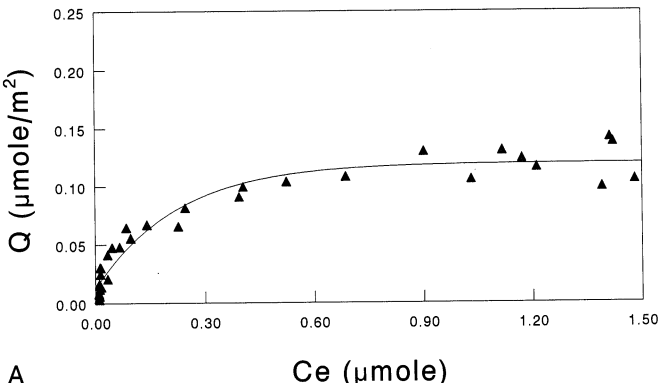
The effect of CPLX-PS on HA seeded growth was monitored after coating synthetic apatite preparations with the concentrations of CPLX-PS calculated from the adsorption isotherm, to coat 0, 30, 60, and 120% of the total apatite surface. Coating was accomplished by incubating the apatite preparation in the pH 7.4 buffered NaCl solution, as described for the adsorption isotherms, except that in this case no radioactive tracer was included. The coated seed crystals were dried by desiccation, ground to a fine powder, and suspended in a metastable calcium phosphate solution, initially containing 1.5 mM $CaCl_2$ and 1.5 mM Na_2HPO_4 in 0.15 M Tris buffer (pH 7.4). Tubes were incubated at 37°C. Aliquots were withdrawn at 0, 1/2, 1, 2, 4, 6, 8, and 24 hours, centrifuged to separate the mineral crystals, and the Ca [27], and phosphate [28] contents of the supernate were determined. First-order rate constants for the disappearance of Ca were determined using data for the first 4 hours. Data were plotted as log [Ca] versus times, and the slope (rate constant) was measured by linear regression.

Preparation of Liposomes

Liposomes were made using a modification of the technique described by Eanes et al. [34]. One-half of the Negative Liposome Kit of Avanti (Avanti, Birmingham, AL) consisting of 4.5 μm cholesterol, 9 μm dicetyl phosphate, and 32 μm egg phosphatidylcholine (PC) were used. To test the influence of PS and CPLX-PS, the phospholipid content of the liposomes was altered by replacing a fraction of the PC with an equivalent molar amount of PS or CPLX-PS. The lipids, dissolved in chloroform:methanol (2:1), were rotary evaporated to a thin film in vacuo at room temperature, and resuspended with 1 ml of a 20 mM phosphate buffer containing 125 mM NaCl under nitrogen. It was vortexed (5 minutes) and ultrasonicated (Model W225 Ultrasonic Processor (Heat Systems, NY)) with a microprobe tip, under a stream of N_2 in an ice bath for 30 minutes and in 3-minute intervals. The liposomes were transferred to a 60 cm (effective length) by 1.1 cm (internal diameter) column (Ace Glass Inc, Vineland NJ) containing 3.1 g Sephadex, bead size 50–150 μm which had been suspended in deairated 2.9 mM sodium phosphate buffer containing 117 mM NaCl. The gel occupied about 60% of the column volume. Fractions of 1.3 ml were collected at a flow rate of 0.4–0.7 ml/minute A 5,6-carboxy fluorescein marker was added to the liposomes until the pattern of elution was established. The liposomes were consistently eluted in a 2–3 ml band following collection of the 60–65 ml head volume. The content of phospholipids in the liposomes was measured by HPTLC as described above.

Electron Microscopic Evaluation of the Liposomes

Liposome suspensions were diluted 1:10 and placed on formvar-coated grids for examination using a Philips CM-12 transmission electron microscope. Micrographs were taken at random at five different sites on the grid. At least 30 different liposomes were measured per grid, and the mean and standard deviation of the observed diameters were calculated.



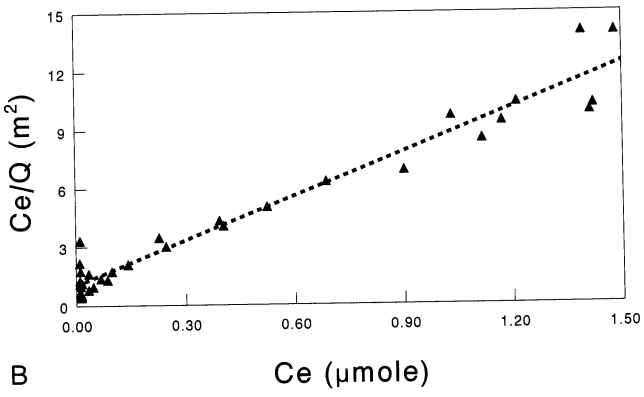


Fig. 1 (A). Binding of ^{3}H-labeled CPLX-PS to hydroxyapatite at 37°. Q, the amount of CPLX-PS bound/m^2 HA, is plotted as a function of the equilibrium concentration, Ce, for CPLX-PS. **(B)** Linearized version of the adsorption isotherm in **(A)** shows Ce/Q versus Ce. The line, determined by linear regression, has a slope of 1/N and an intercept of 1/NK, where N is the number of binding sites and K the affinity constant.

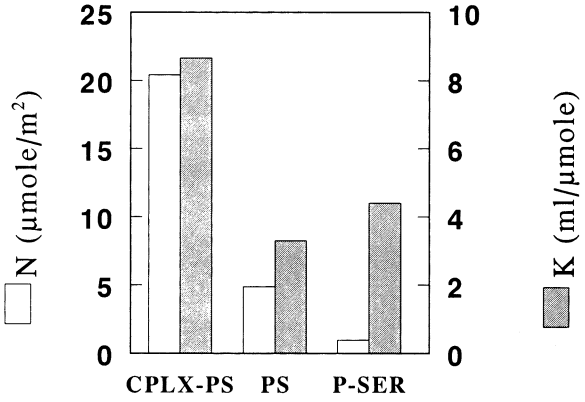


Fig. 2. Comparative affinity constants (K) and number of binding sites (N) for CPLX-PS, PS, and the amino acid phosphoserine (P-Ser) on hydroxyapatite. Data for PS and phosphoserine taken from references 26 and 33, respectively.

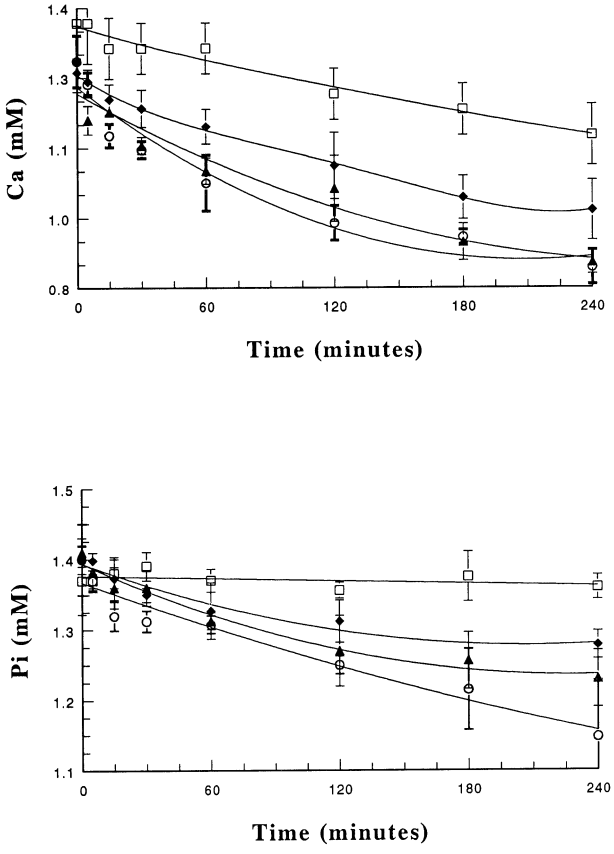


Fig. 3. Effect of CPLX-PS on HA seeded growth is revealed by the concentration-dependent decrease in calcium (top) and inorganic phosphate (bottom) concentrations. Data (mean ± SD) are shown for 0 (open circles), 30 (closed triangles), 60 (closed diamonds), and 120 (open squares) % of the HA surface coated, as estimated from the Langmuir adsorption isotherm.

Mineralization Assays

Liposome-associated mineralization was measured by a modification of the method described by Eanes et al. [34]. The liposomes, containing 1–6 μmol of total phospholipid per ml, were diluted with calcium and phosphate solutions such that the final CaxPi product of the final suspension was 2.25 mM Ca × 1.4 mM Pi and the liposome phospholipid concentration 0 (liposome-free controls) to 3 μmol/ml. The liposomes were made permeable to calcium by the addition of 20 μl of 0.6 mM/ml of the ionophore A23187 in ethanol. To monitor the rate of mineralization, changes in calcium content [27] were sampled from time = 0 (on addition of the ionophore), with triplicate measurements at 1/2 hour, 1, 2, 4, 6, 8, 24, 26, and 48 hours. Additional controls received liposomes but no ionophore.

Enzyme Kinetics

The procedure described by Dennis [35] was modified to monitor the effects of the enzyme phospholipase A_2 on the hydrolysis of PS, free or in CPLX-PS. In brief, the phospholipid was suspended in 2 ml of a solution containing 100 mM of Triton and 1 mM $CaCl_2$. The pH was adjusted to 7.40 using a Brinkman pH Stat with 0.001 N NaOH as titrant, and the reaction was maintained at 37°. After the pH had remained at 7.40 for at least 30 minutes, an aliquot (100 μl) was removed for measurement of organic phosphate substrate concentration, and 2.5 μl, of 1 μ/μl of phospholipase A_2 was added. The activity of each batch of enzyme used was verified using dipalmitoyl-phosphatidylcholine as substrate.

Table 2. Effect of CPLX-PS coating on HA-seeded growth

Surface-coated (%)	k_{Ca} [a]	r
0	0.088 ± 0.006	0.98
30	0.079 ± 0.008	0.92
60	0.058 ± 0.004[b,c]	0.97
120	0.039 ± 0.005[b,c]	0.88

[a] First order rate constant for disappearance of calcium, mean ± SD for four independent experiments; based on linear regression of logarithmic data up to 4 hours
[b] Significantly different from 0 coating, $P \leqslant 0.001$, Tukey-Kramer multiple comparison test
[c] Significantly different from previous value, $P \leqslant 0.01$, Tukey-Kramer multiple comparison test

The rate of the reaction was monitored as the rate of addition of titrant, with initial rates calculated from the linear portions of the curves. Reaction kinetics were calculated assuming the hydrolysis followed a Michaelis-Menton model.

Results

Table 1 (section 1) summarizes the composition of five batches of synthetic CPLX-PS used for these studies. As previously reported, the composition of the CPLX-PS was variable [2, 36]. The mean molar ratio of Ca to total P in this group of samples was 0.7:1, though it had earlier been nearer to 1:1. This discrepancy is probably caused by the higher inorganic phosphate level due to the different technique used to separate and measure it.

Table 1 (section 2) traces the distribution of labeled PS in the various steps during the synthesis of CPLX-PS. The table demonstrates that of the starting PS, only 10% was not incorporated into CPLX-PS. Further, only 30% of the starting PS was lost during the isolation and purification procedures.

The synthetic CPLX-PS bound with relatively high affinity, but low specificity to hydroxyapatite. Figure 1A shows the Langmuir adsorption isotherm, and includes the linearized data from which the binding affinity constant and number of binding sites were calculated (B). The affinity constant, K, was 8.66 ml/μmole, and the number of binding sites, N, 20.4 μmole/m^2 (Table 1, section 3). These data are summarized in Figure 2 along with previously published results for phosphoserine [33] and phosphatidylserine [26]. This figure shows that the number of binding sites for CPLX-PS on apatite is relatively high, indicative of nonspecific binding, and the affinity constant is also relatively high.

When HA seed crystals were coated with CPLX-PS such that 0–120% of the possible binding sites were covered, there was a dose-dependent inhibition of crystal growth as measured by the rate of decrease of Ca (Fig. 3, top) and phosphate (Fig. 3, bottom) in the solution. Table 2 describes the first order rate constants for the disappearance of Ca from solution calculated from these data. As previously reported [6], and confirmed in data not shown here, comparable concentrations of CPLX-PS in the absence of seeds promoted mineral deposition.

The liposomes synthesized with PC only, and those containing PC and CPLX-PS had similar characteristics when examined by electron microscopy. The average diameter of the liposomes containing PC only was 380 ± 141 nm, whereas the average diameter of liposomes with 85 mole% of the phospholipid as PC and 15 mole% as PS in CPLX-PS was 388 ± 97 nm.

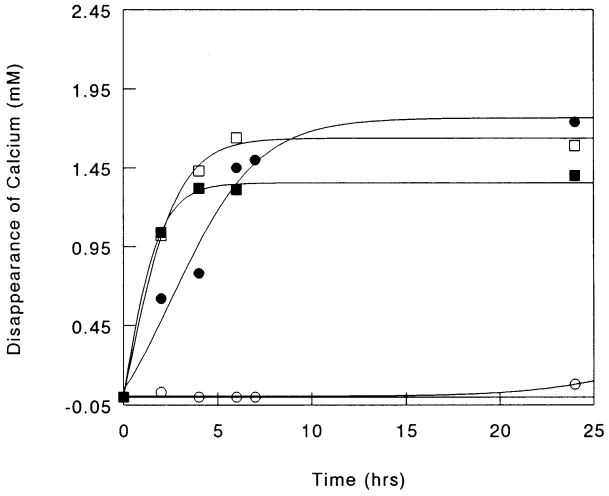


Fig. 4. Two typical experiments comparing the changes in solution Ca concentrations in control liposomes in which 18 mole% of the PC was substituted with PS (closed symbols) and 10 mole% of PC was substituted with CPLX-PS (open symbols). Time 0 represents the time of addition of ionophore. (●) PC exp 52; (○) 18% PS exp 52; (■) PC expt 57; (□) 10% CPLX-PS exp 57.

The rate of liposome-mediated mineralization was influenced by several factors, one of which was the concentration of liposome in the solution. The data presented here are limited to experiments that contained 1.5 μmol/ml of phospholipid. Figure 4 illustrates the effect of replacing fractions of the PC with PS or CPLX-PS in two separate experiments along with their PC only controls. When 10% of the PC was replaced with CPLX-PS, the uptake of calcium was enhanced relative to the PC only control, whereas when 18% of the PC was replaced with free PS, complete inhibition of mineralization occurred.

Figure 5 summarizes the effects of replacing part of the PC with different amounts of PS or CPLX-PS in all experiments that had a total of 1.5 μmol phospholipid/ml. Because of the variability of the rate at which mineralization occurred, and because results were more consistent at the 24-hour sampling than at earlier times, the data is presented as the ratio of the 24-hour change in calcium for the tubes containing PS or CPLX-PS compared to that for their respective PC only controls. As seen in this figure, when 15–30% of the PC was replaced with free PS, mineralization was completely inhibited. On the other hand, substitution of PC by CPLX-PS in high concentrations was partially inhibitory relative to liposomes with PC alone, and either equivalent to PC, or stimulatory at lower concentrations.

Figure 6 illustrates the relative resistance of CPLX-PS to hydrolysis by phospholipase A_2. This Michaelis-Menton type plot presents data for PS and CPLX-PS. The Km values were 4.63 mM for CPLX-PS and 0.27 mM for PS with V_{max} equal to 0.029 ml/minute for CPLX-PS and 0.066 ml/minute for PS. As is seen from this data, CPLX-PS is not as good a substrate for this enzyme as free PS.

Discussion

The results of this study provide an explanation for the persistence of CPLX in the mineralizing tissues. First, the

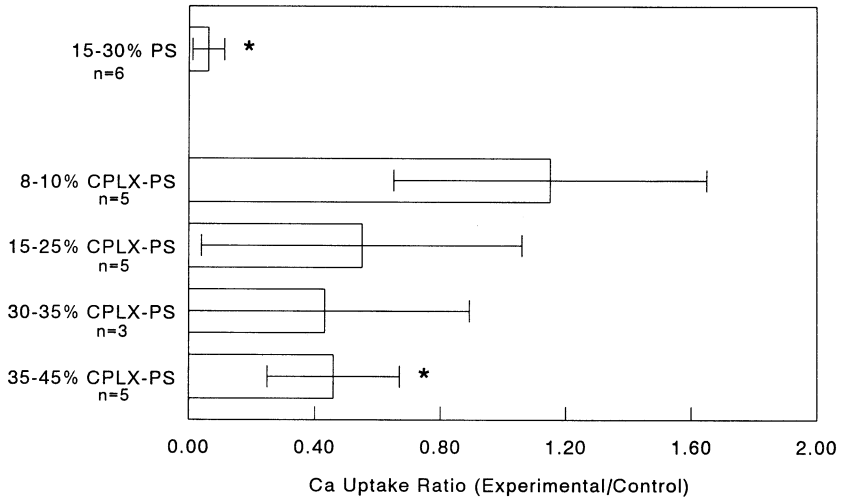


Fig. 5. Effect of CPLX-PS and PS on liposome-mediated mineralization is revealed by the ratio of the change in Ca in its respective PC-only control liposomes in the experimental liposomes (with CPLX-PS or PS substituted for PC) to the change in Ca in the PC-only control liposomes at 24 hours. Values for similar liposome compositions were averaged and are shown as mean ± SD, with n indicated. *Indicates that ratio is different from one ($P \leqslant 0.01$).

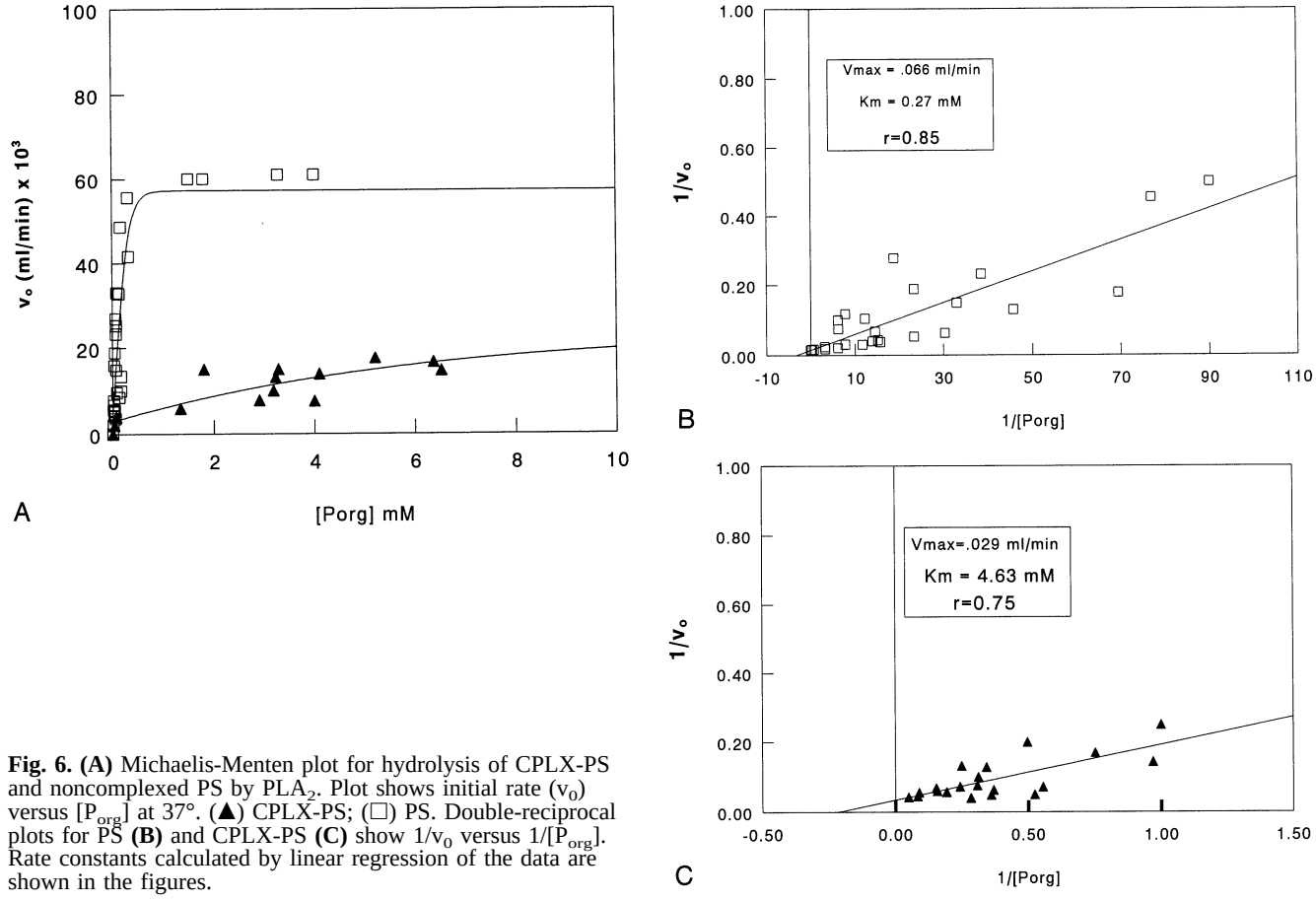


Fig. 6. (A) Michaelis-Menten plot for hydrolysis of CPLX-PS and noncomplexed PS by PLA_2. Plot shows initial rate (v_0) versus $[P_{org}]$ at 37°. (▲) CPLX-PS; (□) PS. Double-reciprocal plots for PS **(B)** and CPLX-PS **(C)** show $1/v_0$ versus $1/[P_{org}]$. Rate constants calculated by linear regression of the data are shown in the figures.

synthetic CPLX-PS was shown to bind relatively nonspecifically to HA. Based on the calculated affinity constants it can be estimated that a proportion of CPLX in calcified cartilage may be there because of this affinity. Second, the results demonstrated that even when bound to HA or incorporated into liposomes, CPLX-PS as distinct from free-PS can still facilitate mineral deposition. Finally, the resistance of CPLX-PS to attack by phospholipase A_2 explains why complexed acidic phospholipids can persist in a tissue with high phospholipase A_2 activity.

Synthetic complexes of Ca and inorganic phosphate with phosphatidylserine bound to HA with high affinity and low specificity, compared with the amino acid phosphoserine [33] and the free phospholipid phosphatidylserine [26]. Because of this high affinity, CPLX-PS can retard growth of apatite seed crystals, by blocking growth sites, although the extent of retardation was appreciably less than that reported for equivalent amounts of PS, or equivalent number of sites coated with PS [26]. This may be due to the ability of bound CPLX-PS to dissociate from HA and continue to facilitate

mineral deposition, or to the nature of the interaction between CPLX-PS and the mineral.

Based on the amount of CPLX in the hypertrophic cell zone of the bovine growth plate and the mineral content of that zone [11], one can calculate that there is approximately 9.5 mg CPLX/mg mineral. Assuming a molecular weight for CPLX of 1×10^3, and a specific surface of the mineral of 100 m^2 [37], this would correspond to 95 CPLX μmole/m^2 of calcified cartilage which is significantly higher than the value indicated by the number of binding sites calculated for CPLX-PS. Although several assumptions had to be made to do this calculation, and the actual specific surface of the cartilage mineral has not been measured, these values can be interpreted as suggesting that much of the CPLX in this tissue is present because of CPLX binding to initially formed mineral. However, there must also be CPLX associated with cell or matrix vesicle membranes, as originally demonstrated by Wuthier and Gore [19].

An equally important explanation for the accumulation of CPLX in the hypertrophic cell zone is the resistance of CPLX to hydrolysis by phospholipase A_2. Although this enzyme's activity in the growth plate is maximal in the hypertrophic cell zone [20], since CPLX-PS is a poor substrate for the enzyme, it is likely that CPLX is not removed by this enzyme activity as the growth plate becomes mineralized. This also accounts for the earlier observation of increased PS content in this zone [38] since some of the PS measured may have been part of the CPLX. Further, it is likely that CPLX continues to play a role in the mineralization process. As shown by this study, if the presence of CPLX does not markedly block mineral crystal proliferation, the accumulation of CPLX in the calcifying tissues would not be detrimental to the accumulation of new mineral.

Wu et al. [9] recently demonstrated that the ''nucleational core'' in extracellular matrix vesicles consisted of annexins (proteolipids) and PS associated with Ca and P, i.e., CPLX-PS. Liposomes have been extensively used as models of matrix vesicle-mediated calcification [22–25, 34]. But, when inserted into liposomes, PS blocks liposome-mediated apatite deposition [22, 23]. However, as shown in this study, when CPLX-PS is inserted into similar liposomes on a mole:mole basis in place of noncomplexed PS, mineral formation is not markedly retarded, and may actually be promoted. This is in agreement with the view that CPLX plays a major role in matrix vesicle-mediated calcification.

Although the liposome experiments clearly demonstrated the inhibitory effects of free PS, the results with PC alone, and CPLX-PS substituted for 4–45% of the total PC were more variable. The reason for the variability in the liposome experiments is uncertain. In the case of the PC-only liposomes, the lack of reproducibility may reflect variations in the total amount of inorganic phosphate incorporated into the liposome, variations in leakage from the liposomes, changes in the rate at which the ionophore was delivered to the liposome, or alterations in other liposome properties. When CPLX-PS was added to the liposomes in some experiments, mineralization was greater than that in liposomes with PC only, whereas in others, there was slight inhibition or no effect. This appears to be the analog of the seeded apatite growth studies in which coating of apatite surfaces with CPLX-PS was found to retard apatite growth, but not completely inhibit it. Since the major changes in solution composition occur after the HA crystals have penetrated the liposome and are exposed to the external solution [34], once the first crystals have formed in the liposome, the extent of coating by either PS or CPLX-PS may affect their rate of growth and the length of time before exposure to the external solution. Thus, lower concentrations of CPLX-PS may serve as mineralization promoters, but have no ability to coat the initial HA crystals. Higher concentrations may also facilitate the initial formation of HA crystals, but would then be available to coat the crystals and block their proliferation.

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