The Divalent Cation Is Obligatory for the Binding of Ligands to the Catalytic Site of Secreted Phospholipase A_2[†]

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ABSTRACT: The divalent cation requirement for partial reactions of the catalytic turnover cycle during interfacial catalysis by pig pancreatic phospholipase A_2 (PLA2) is investigated. Results show that the specific role of calcium in all the events of the catalytic cycle at the active site is not shared by other divalent cations. Cations such as calcium, barium, and cadmium bind to the enzyme in the aqueous phase. The active-site-directed ligands (substrate, products, and transition-state mimics) do not bind to the enzyme in the absence of a divalent cation. The synergistic binding of such ligands to the active site of PLA2 bound to the interface is, however, observed only in the presence of isosteric ions like calcium and cadmium, but not with larger ions like strontium or barium. The equilibrium constants for ligands bound to the enzyme in the presence of calcium and cadmium are virtually the same. However, only calcium supports the catalytic turnover; the rate of hydrolysis in the presence of cadmium is less than 1% of that observed with calcium. *The role of divalent ions on the interfacial catalytic turnover cycle of PLA2 is not only due to the cation-assisted binding of the substrate but also due to its participation in the chemical step.* Other roles of divalent ions in the events of interfacial catalytic turnover are also identified. For example, the binding of the enzyme to the interface is apparently promoted because the divalent cation is required for the sequential step, i.e., the binding of the substrate to the active site of PLA2. Apparent activation of PLA2 also occurs by nonspecific effects related to the effect of cations on the organization of the substrate interface; for example, cations increase the apparent rate of hydrolysis by promoting the rate of substrate replenishment on the enzyme-containing particles by inducing the intervesicle exchange of the enzyme (Jain et al., 1986b) or by promoting fusion of vesicles (Jain et al., 1986a).

Calcium is an obligatory cofactor for interfacial catalysis by secreted phospholipase A_2 (PLA2)[1] from virtually all sources, including pancreatic juices, snake and bee venoms, and inflammatory exudates. It is required for the catalytic activity on all forms of substrate interfaces (Verheij et al., 1982). Binding of calcium to the conserved Asp-49 in the active site region has been qualitatively demonstrated by covalent modification studies (Verheij et al., 1979; Fleer et al., 1981), by site-directed mutagenesis (van den Bergh et al., 1988, 1989), and by X-ray diffraction studies on single crystals of PLA2 (Dijkstra et al., 1981; Renetseder et al., 1985). The requirement of calcium as a cofactor for the binding of the substrate analogues to the active site has been shown by spectroscopic (Slotboom et al., 1978), covalent modification (Verheij et al., 1980; Jain et al., 1991a), and X-ray diffraction studies (Scott et al., 1990; Thunnissen et al., 1990). It is also implicit in all the discussions of the mechanism of the PLA2 action (Verheij et al., 1980; Scott et al., 1991) that the primary role of the calcium ion is putatively in the electrophilic catalysis, i.e., the polarization of the carbonyl group of the *sn*-2-ester linkage of the substrate.

The kinetic evidence for the role of calcium in interfacial catalysis by PLA2 has not been resolved in terms of the primary rate and equilibrium parameters. Interfacial catalysis is a complex process involving not only the steps of the catalytic turnover cycle in the interface but also the steps involved in the interaction of the enzyme to the interface (Verheij et al., 1982; Jain & Berg, 1989). Analytical formalisms and experimental protocols designed to dissect the primary kinetic and equilibrium parameters of interfacial catalysis by PLA2 (Berg et al., 1991; Jain et al., 1991a; Dupureur et al., 1992a,b) are used in the present study to establish the role of divalent cations for the binding of competitive inhibitors and products to the active site of PLA2. The overall role of a divalent cation can be accommodated in the general framework of the kinetic scheme for interfacial catalysis (Jain & Berg, 1989; Berg et al., 1992). This scheme is elaborated further to account for the role of divalent cations in terms of the equilibria shown in scheme III in Figure 1. Since catalysis by PLA2 occurs substantially, if not exclusively, at the interface (Rogers et al., 1992), we have considered only the interfacial processes. Results described in this paper demonstrate that most divalent cations bind to the enzyme in the aqueous phase, whereas only calcium and cadmium support the binding of the active-site-directed ligands.

EXPERIMENTAL PROCEDURES

All studies described in this paper were carried out with PLA2 prepared from pig pancreas or other sources as described (Jain et al., 1991b). DMPM (Jain et al., 1986a; Jain & Gelb, 1991), DTPM (Jain et al., 1986a), and deoxy-LPC and MJ33 (Jain et al., 1991d) were synthesized as described. Inhibitors of the MG series were provided by Professor Michael Gelb

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[1] Abbreviations: deoxy-LPC, 1-hexadecyl-*sn*-3-glycerophosphocholine; DMPC, 1,2-dimyristoyl-*sn*-3-glycerophosphocholine; DMPM, 1,2-dimyristoyl-*sn*-3-glycerophosphomethanol; DTPM, 1,2-ditetradecyl-*sn*-3-glycerophosphomethanol; MG14, 1-octyl-2-(phosphonoheptyl)-*sn*-3-glycerophosphoethanolamine; MJ33, 1-hexadecyl-3-(trifluoroethyl)-*sn*-2-glycerophosphomethanol (racemate was used); PLA2, phospholipase A_2 from pig pancreas, unless indicated otherwise.

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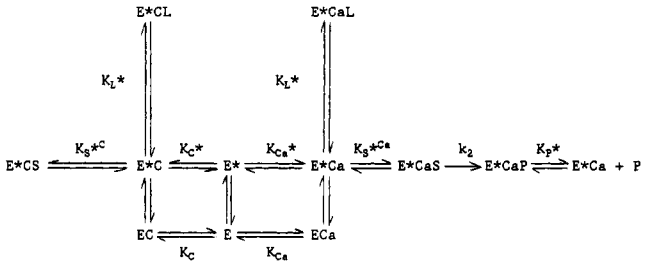

FIGURE 1: Scheme III for interfacial catalysis which elaborates the cation-dependent equilibria for the binding of the ligand and substrate to the enzyme at the interface. This is an expanded version of the minimal kinetic schemes I and II developed in Jain and Berg (1989) and Berg et al. (1991). The experimentally demonstrated key feature of scheme III is that the binding of the cation to the enzyme leads to the formation of the E*S complex, as with calcium, which undergoes the chemical change. E*L can also be a dead-end complex, as with cadmium. On the other hand, EC (as with barium or strontium) could remain in the aqueous phase, without the formation of the E* and E*L species. All possible equilibria in the aqueous phase are not developed in this scheme; also the hydrolysis of solitary monomeric substrate in the aqueous phase also appears unlikely (Rogers et al., 1992).

(Seattle). (R_P)- and (S_P)-DPPsC were provided by Dr. Carol Bruzik (Columbus, OH). DMPC was from Avanti.

Kinetic analysis of the hydrolysis of sonicated DMPM (typically 0.3 mM) vesicles in the scooting mode by PLA2 was carried out as described previously using a pH-stat method at pH 8.0 and 23 °C in a 4-mL reaction mixture (Jain et al., 1986a, 1991a,c). Reaction was initiated by adding PLA2 to the mixture preequilibrated in a stream of nitrogen. The reaction mixture for the first-order progress curve (Jain et al., 1986a, 1991c; Jain & Gelb, 1991; Berg et al., 1991) contained 0.5 mM $CaCl_2$, 1 mM NaCl, and 0.1–0.4 μg of PLA2. For progress curves under the zero-order conditions (Berg et al., 1991, 1991c) the reaction mixture contained 1 mM $CaCl_2$, 0.1 M NaCl, 20 μg of polymyxin B sulfate, and 0.02–0.1 μg of PLA2. Hydrolysis of DMPC (1 mM) vesicles was also monitored by the pH-stat method under similar conditions in the reaction mixture containing 6 mM $CaCl_2$, 1 mM NaCl, 0.02–0.1 μg of PLA2 at 23 °C and pH 8.0, and 0.15 mM deoxycholate or 0.15 mole fraction of the products of hydrolysis. Studies in the presence of other divalent ions were carried out either by adding a small volume of the concentrated stock solution (typically 50 mM) while the reaction was in progress or by adding the cation solution before adding the enzyme. The order of addition of the cation was not critical, except for the pH drop caused by their hydrolysis or interaction with DMPM vesicles. In the presence of certain cations, formation of insoluble hydroxides can create significant difficulties. Since the solubility product of $Cd(OH)_2$ is 1.2×10^{-14} M, anomalous behavior is expected at pH 8.0 if the cadmium concentration exceeds 0.01 mM. On the basis of such considerations at pH 8.0, cations like cadmium could be used below 0.01 mM in the presence of DMPM vesicles. Some difficulties with traces of other divalent cations were also encountered. Typically for low calcium runs before calcium was added, the reaction mixture was filtered through a regenerated column of "Chelex" to remove traces of multivalent cations. The background calcium under these conditions was estimated to be less than 10 μM. The background rate of hydrolysis of DMPM in the presence of 0.05 mM EGTA was less than 0.05 s^{-1}.

Determination of Dissociation Constants by the Protection Method. As described in detail elsewhere [Scrutton and Utter, (1965), with modifications described in Jain et al. (1991a)], the equilibrium constants for the dissociation of cations or active-site-directed ligands (inhibitors, products, or the ether analogue DTPM) bound to the active site of PLA2 at the interface of a neutral diluent (deoxy-LPC) or in the aqueous phase were determined by monitoring the rate of alkylation of His-48 by 2-bromo-4-nitroacetophenone (*p*-nitrophenacyl bromide). Briefly, 0.03 mL of the incubation mixture at 22 °C and pH 7.3 contained 0.4 μM PLA2, 50 mM cacodylate buffer, 2 mM *p*-nitrophenacyl bromide, 0.035 mg of γ-globulin, 1.6 mM deoxy-LPC, and ligands including divalent ions at appropriate concentrations or mole fractions. At various intervals, an aliquot containing 0.01–0.1 pmol of enzyme was diluted into the assay mixture (Jain et al., 1991c; Radvanyi et al., 1989). The fraction of the enzyme that remained unmodified in the original incubation reaction mixture was monitored most conveniently by adopting the fluorescence-based protocol described by Radvanyi et al. (1989). Typically, an aliquot of the incubation mixture was added to the stirred fluorescence assay mixture containing a sonicated dispersion of 1 μg of 1-palmitoyl-2-(pyrenyldecanoyl)-*sn*-3-glycerophosphomethanol (Molecular Probes), 0.25 mg of bovine serum albumin, 100 mM NaCl, 0.25 mM $CaCl_2$, and 50 mM Tris·HCl at pH 8.5 and 23 °C. The change in the emission fluorescence at 395 nm (excitation at 345 nm with both slit widths at 4 nm each) was continuously monitored on a SLM4800S spectrofluorometer with output to a chart recorder. Independent controls were carried out to demonstrate the linearity of the fluorescence change as a function of the enzyme concentration. Also different batches of serum albumin showed very different behavior, especially for the stability of the baseline in the absence of the enzyme; therefore, it was necessary to test batches from different suppliers till a suitable batch was identified.

The residual phospholipase A_2 activity in the inactivation assay was determined from the initial rate of hydrolysis observed in the fluorescence assays. The nonlinear regression plot of the residual PLA2 activity as a function of time provided the rate constant for inactivation. The equilibrium dissociation constant under a given set of conditions was calculated from the half-times for the inactivation (Jain et al., 1991a):[2]

$$\frac{t_L}{t_L - t_0} = \frac{1 + K_L^*/X_L}{1 - k_L/k_0} \quad (1)$$

Here t_0 is the half-time for the inactivation of E* by an alkylating agent in the absence of a ligand, and t_L is the half-time in the presence of a ligand at mole fraction or concentration X_L. Under all the conditions reported in this paper, the ratio of the intrinsic inactivation rate constants for E*L and E* with the alkylating agent, k_L/k_0, was <0.03 with saturating mole fractions of all the active-site-directed ligands.

[2] Definitions of the kinetic and equilibrium parameters: All constants with an asterisk represent values for the equilibrium dissociation constants for the reaction in the interface. It should be noted that some of the notations with an asterisk correspond to those without asterisks used previously (Berg et al., 1991). The equilibrium dissociation constants of the divalent cation bound to PLA2 as shown in Figure 1 are defined as follows: K_C, for EC in the aqueous phase; K_C*, for E*C at the interface; K_C*(L), effective dissociation constant for a cation at the subsaturating concentration of the ligand; K_L*, dissociation constant of a ligand L, such as an inhibitor (I) or products (P), at the saturating concentration of the cation; K_L*(C), effective dissociation constant for a ligand at the given cation concentration; K_M*, Michaelis constant for the catalytic turnover in the interface; k_{cat}, turnover at the saturating substrate concentration in the interface; N_S, the number of phospholipids in the outer monolayer of the vesicle; $N_S k_i$, apparent second-order rate constant for the catalytic turnover in the interface in the scooting mode; v_0, turnover number at mole fraction of the substrate $X_S = 1$.

The dissociation constant for a ligand bound to PLA2 was calculated from the plots of $t_L/(t_L - t_0)$ versus $1/X_L$. For example K_{Ca}*, the dissociation constant for calcium bound to PLA2 at the interface of deoxy-LPC, was obtained from the half-times for alkylation of PLA2 bound to deoxy-LPC in the absence (t_0) or presence (t_L) of a known concentration of calcium. Dissociation constants for the water-soluble ligands are expressed in bulk concentration units (mM), whereas the K_L* values of ligands dispersed in the interface of a neutral diluent are expressed in mole fraction units.

The binding of the active-site-directed ligands was observed only in the presence of certain divalent cations; i.e., the binding of the cation to the enzyme is obligatory before the ligand can bind. For such obligatory equilibrium the effective dissociation constant for the cation, K_C*(L), was obtained from the half-time in the absence of calcium (t_0) and the half-time in the presence of calcium and ligand (t_{CL}) at mole fraction X_L. K_C*(L) is related to the dissociation constant of the cation in the absence of the ligand, K_C*, by

$$\frac{t_0[C]}{t_{CL} - t_0} = K_C^*(L) = \frac{K_C^*}{1 + X_L/K_L^*} \quad (2)$$

The first equality is based on eq 1 and the fact that $k_L/k_0 = 0$. Thus, K_L* is the dissociation constant for the ligand at saturating calcium (or other divalent cation) concentration. It is related to the effective dissociation constant of the ligand, K_L*(C), at a given concentration of the divalent ion, [C], by

$$\frac{t_C X_L}{t_{CL} - t_C} = K_L^*(C) = K_L^*\left(1 + \frac{K_C^*}{[C]}\right) \quad (3)$$

K_L*(C) was obtained from the half-time in the presence of calcium (t_c) and the half-time in the presence of calcium and ligand (t_{CL}) at mole fraction X_L. K_C* can be obtained directly by monitoring the inactivation times in the absence of other active-site-directed ligands. Also on the basis of eq 2 or 3, K_L* and K_C* can be obtained from the linearized plots of the effective dissociation constants K_C*(L) or K_L*(C) as a function of X_L or [C]. Results obtained by both protocols were virtually the same, and were consistent with the value obtained from the kinetic studies. The standard deviation in the values of the dissociation constants reported in this paper is less than 30%.

Spectroscopic Methods. Binding of PLA2 to the interface and the binding of a ligand to the enzyme at the interface exhibit characteristic changes in the absorbance or the fluorescence spectrum. The change in the absorbance of PLA2 in the 240–340-nm region was monitored by a UV–vis spectrophotometer (Hewlett-Packard Model 8452) equipped with a diode array detector. The standard software package from the supplier allowed manipulations that were necessary for obtaining the difference spectrum. The resolution was 2 nm, and therefore peaks in the difference spectrum appear sharper. Typically, the PLA2 concentration was 35 μM in 20 mM Tris·HCl at the indicated concentration of the cation, ligand, and the neutral diluent. The first addition was usually of the neutral diluent, and then appropriate amounts of ligand or divalent ion were added in small volumes such that the overall dilution was less than 5%. Under certain conditions minor scattering contributions from micelles were observed, which could be appropriately subtracted. The spectra were also corrected for dilution before subtractions were made for obtaining the difference spectra.

The fluorescence change was monitored as a change in the emission intensity at 335 nm (excitation at 290 nm) with slit widths of 4 nm each on a SLM 4800S spectrofluorometer with a 450-W xenon lamp. The reaction mixture (1.5 mL) contained 2–7 μM PLA2 in 10 mM Tris·HCl and the indicated final concentrations of the cation, ligand, and neutral diluent. Binding of the various ligands was characterized as described in the text and figure legends. The nonlinear fit of the titration data to a rectangular hyperbola gave values of apparent or effective dissociation constants. With suitable corrections the spectroscopic methods could also be used for the determination of the equilibrium constants. Although these results and the relevant controls are not described in this paper, the validity of this method is shown elsewhere (Dupureur et al., 1992a,b).

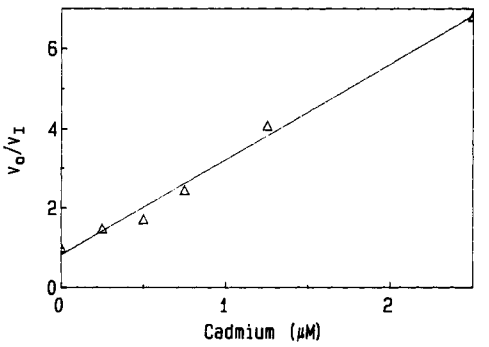

FIGURE 2: Effect of cadmium on the relative initial rate of hydrolysis of DMPM (0.16 mM) vesicles in the presence of 1 mM $CaCl_2$, 0.1 M KCl, and 5 μg/mL polymyxin B sulfate by pig pancreatic PLA2.

Table I: Equilibrium Dissociation Constants and IC(50) Values (mM) for the Inhibition of the Hydrolysis of DMPM Vesicles by Pig Pancreatic PLA2 at 23 °C, pH 8.0, and [Ca] = 1 mM

cation	r (Å)	IC(50)	K_c*(S)	K_c
Ca^{2+}	0.99			0.33
Cd^{2+}	1.00	0.001	<0.0005	0.16
Sr^{2+}	1.13	0.68	0.17	0.16
Ba^{2+}	1.35	0.85	0.44	0.32
Mg^{2+}	0.72	>2.0		>2
Gd^{3+}	1.00	0.025	0.0025	0.01
Tb^{3+}	0.98	0.0036	0.001	0.021

RESULTS

Inhibition of Interfacial Catalysis by Divalent Cations. Calcium is a catalytic cofactor for PLA2. Therefore, other divalent cations which compete with calcium for the binding site would change the rate of hydrolysis by PLA2. Indeed, the rate of hydrolysis of DMPM vesicles in the scooting mode is inhibited immediately after the addition of certain divalent cations to the reaction mixture. For example, the cadmium concentration dependence of the relative rate of hydrolysis by PLA2 in the presence of $CaCl_2$ is shown in Figure 2. The concentrations of the cadmium required for 50% inhibition of the steady-state rate of hydrolysis, i.e., IC(50) values, obtained from such plots for several cations are summarized in Table I. These results show that the inhibitory effect of cations on the hydrolysis of DMPM vesicles was quite specific. Cadmium is one of the most effective inhibitors of the hydrolysis of DMPM by PLA2 in the scooting mode with an IC(50) of 0.001 mM compared to >0.5 mM for barium and strontium. As described elsewhere, monovalent ions had no noticeable inhibitory effect on the rate of hydrolysis (Jain et al., 1986b, 1991c). Also magnesium had little effect on the rate of hydrolysis, whereas terbium and gadolinium were strongly inhibitory.

On the basis of the observation that in the presence of excess cadmium the rate of hydrolysis was <1% of that observed with 1 mM calcium alone, the possibility that

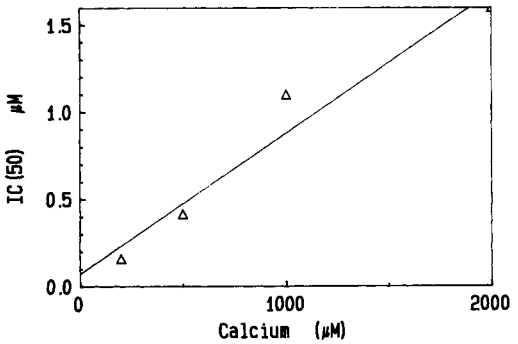

FIGURE 3: IC(50) for cadmium versus [Ca] for the hydrolysis of DMPM vesicles (eq 4). Other conditions are as given for Figure 2.

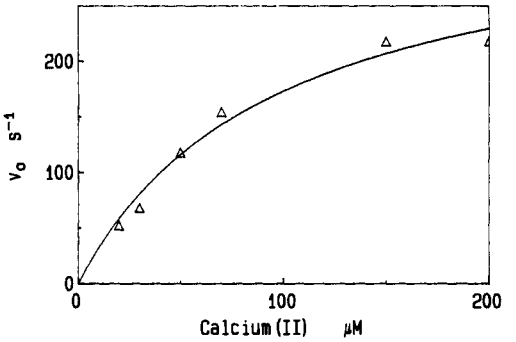

FIGURE 4: Dependence of the initial rate of hydrolysis (v_0) of DMPM vesicles on the calcium concentration. Other conditions are as in the legend to Figure 2. The curve is a theoretical fit for $K_{Ca}^*(S) = 0.11$ mM and maximum $v_0 = 300$ s^{-1}.

cadmium competes with calcium for the enzyme during the catalytic turnover was tested as follows. As shown in Figure 3, the IC(50) value for cadmium depends on the concentration of calcium in the reaction mixture. According to the classical Michaelis–Menten formalism (Segel, 1976) adopted for interfacial catalysis (Berg et al., 1991), the effect of a competitive inhibitor on the kinetics of an enzyme-catalyzed reaction is described by the relationship

$$IC(50) = K_C^*(S)\left\{1 + \frac{[Ca]}{K_{Ca}^*(S)}\right\} = K_C^*(S)\left\{1 + \frac{[Ca](1 + 1/K_M^*)}{K_{Ca}^*}\right\} \quad (4)$$

This relationship is derived from scheme III (Figure 1), and it is valid for the competitive inhibition by a cation that replaces calcium for the binding of the substrate but does not support catalysis. It would not be valid for an inhibitor that blocks the substrate binding. It is based on the definition of the effective dissociation constant for calcium under catalytic conditions at a mole fraction of 1 of the substrate, $K_{Ca}^*(S)$. It is related to K_{Ca}^* (right side equality in eq 4), the dissociation constant for calcium bound to the enzyme at the interface in the absence of the ligand (or substrate), as $K_{Ca}^*(S) = K_{Ca}^*/(1 + 1/K_M^*)$. $K_C^*(S)$ as given in eq 2 is the effective dissociation constant for cadmium bound to the enzyme at the substrate interface in the presence of the substrate at a mole fraction of 1, i.e., under the conditions where the IC(50) value is obtained. From the y intercept of the line in Figure 3, the value of $K_C^*(S)$ for cadmium under these kinetic conditions was estimated to be less than 0.0005 mM. Only the upper limit could be estimated because the standard deviation was comparable to the magnitude of the intercept. The slope of the line was $0.0008 = K_C^*(S)/K_{Ca}^*(S)$. This is consistent with the value of $K_{Ca}^*(S) = 0.1$ mM obtained from the calcium concentration dependence of the initial rate of hydrolysis (Jain et al., 1991c), and it is also consistent with the experimentally measured values of K_M^* (=0.35 mole fraction) and K_{Ca}^* (=0.33 mM). These results show that cadmium binds with a high affinity to PLA2 at the DMPM interface. According to eq 2 under these conditions, the effective dissociation constant for cadmium, $K_{Cd}^*(S)$ is related to the dissociation constant of cadmium from the enzyme in the absence of a ligand, K_{Cd}^* as

$$K_{Cd}^*(S) = \frac{K_{Cd}^*}{1 + X_S/K_S^*} \quad (5)$$

Here X_S is the mole fraction of the substrate (=1) under the conditions used for the present studies; K_S^* is the interfacial equilibrium constant for the binding of the substrate at the saturating concentration of the cation, and its value could depend on the nature of the cation. As shown later, the experimentally measured values of K_{Cd}^* (=0.065 mM) and K_S^* (=0.015 mole fraction) are consistent with this relationship.

Cations as Cofactors for the Hydrolysis of DMPM Vesicles in the Scooting Mode. The initial rate of hydrolysis of DMPM vesicles depends upon the calcium concentration. In earlier studies the initial rates at low calcium concentrations could not be measured accurately. Now, with the salt solutions filtered through the "Chelex" column, linear initial rates could be measured at relatively low calcium concentrations. As shown in Figure 4, the calcium concentration dependence of v_0 was fitted to a hyperbola with $K_{Ca}^*(S) = 0.1$ mM compared to a value of 0.16 mM obtained earlier (Jain et al., 1991c) and the maximum rate of hydrolysis at saturating calcium was 300 s^{-1} compared to 270 s^{-1}.

Cadmium and other cations also bind with high affinity to the active site of PLA2, and they support the formation of ternary complexes with the substrate analogs. Therefore, it was of interest to examine the ability of such cations to support the catalytic turnover of DMPM as well as other substrates. Among the cations in Table I, only cadmium showed a detectable rate of hydrolysis of DMPM, approximately 1 s^{-1}, which confirms earlier results with the enzyme from bee venom (Tsai et al., 1985). With other cations the rate was <0.2 s^{-1}. This phenomenon is being investigated further; however, for all practical purposes most cations that were examined were effective inhibitors of hydrolysis in the presence of calcium.

Inhibition of PLA2 from Other Sources. Interfacial catalysis by PLA2 from several other sources was also inhibited by cadmium. The IC(50) values at 1 mM calcium were 0.6 μM for PLA2 from bovine pancreas, 2.2 μM for the Crotalus atrox venom enzyme, 0.8 μM for the human synovial/platelet enzyme, and 0.7 μM for the bee venom enzyme. Essentially all (>95%) of the activity was inhibited at the saturating concentration of cadmium, and the IC(50) values showed little correlation to the K_{Ca}^* values for these enzymes.

Effect of Cations on the Reaction Progress Curve. Elsewhere we have shown that salts promote intervesicle exchange of PLA2 (Jain et al., 1986b). Under these conditions the initial rate of hydrolysis is effectively reduced as the fraction of the enzyme bound to the interface decreases. This possibility was ruled out as an explanation for the inhibitory effect of cadmium. As shown in Figure 5 under the first-order conditions, only the time constant (k_i) for the hydrolysis, but not the extent of hydrolysis per enzyme (N_S), decreases in the presence of cadmium. $N_S k_i$ is the apparent second-order rate constant for the hydrolysis of vesicles under the substrate limiting conditions as is the case on small sonicated vesicles

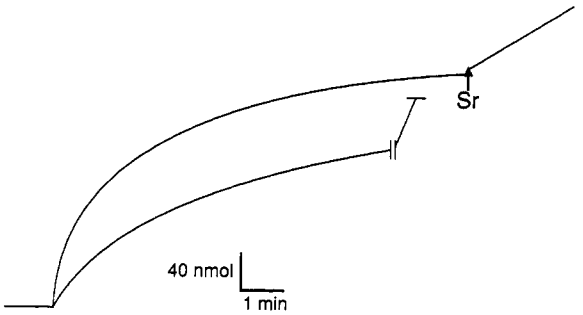

FIGURE 5: Effect of cadmium on the reaction progress curve for the hydrolysis of small sonicated vesicles of DMPM (0.16 mM) by pig pancreatic PLA2 (7 pmol) in a 4-mL reaction mixture containing 0.5 mM $CaCl_2$ and 1 mM NaCl at pH 8.0 (top) without and (bottom) with 0.01 mM $CdCl_2$. The time break in the bottom curve shows that after about 15 min the extent of hydrolysis is the same with and without cadmium. Also shown with the top curve is the fact that, after the reaction has ceased under the first-order conditions, addition of 1 mM strontium initiates a fresh round of hydrolysis as the bound enzyme encounters additional substrate after strontium-induced fusion with excess substrate vesicles [see also Jain et al. (1991c)].

(Berg et al., 1991). From the decrease in the value of $N_S k_i$ with cadmium concentration the value of IC(50) was found to be 1.7 ± 0.2 μM compared to the value of 0.42 ± 0.1 μM obtained from v_0 measurements.

If the bound enzyme exchanged with excess vesicles, the extent of hydrolysis in the presence of cadmium would have been higher. Therefore, it is concluded that the low concentrations of cadmium used in these studies do not promote intervesicle exchange of the enzyme or the fusion of vesicles. This conclusion is also supported by the observation that over a wide NaCl or KCl concentration range (0–1000 mM) the IC(50) value for cadmium did not change significantly (results not shown).

Higher concentrations of divalent cations promote fusion of DMPM vesicles (Jain et al., 1986a). For example, as shown in Figure 5, substrate replenishment caused by the strontium-induced fusion of DMPM vesicles caused an apparent increase in the rate of hydrolysis, even though strontium by itself did not support catalysis (Table I). Such apparent activation due to substrate replenishment strongly depends on the specific assay conditions, and this mechanism accounts for the apparent activating effect of salts and other additives (Jain et al., 1991c).

Inhibition of the Hydrolysis of DMPC Vesicles by Cadmium. Hydrolysis of DMPC vesicles promoted by the products of hydrolysis or deoxycholate is also inhibited by cadmium. From a plot of the type shown in Figure 2, for the hydrolysis of DMPC vesicles IC(50) for cadmium was 3.8 μM in the presence of 1 mM calcium, compared to a value of 1 μM observed for the hydrolysis of DMPM under comparable conditions. These results show that the inhibition by cadmium does not depend on the nature of the substrate; however, the difference between the IC(50) values for these two systems is significant. Although we have not pursued it further, on the basis of eq 4, this difference could be due to a difference in the K_{Ca}*(S) values for the two substrates arising from a difference in the stability of the complexes formed with the two cations. It is also possible that the effective concentration of free cadmium at the interface in the presence of deoxycholate is lower.

Equilibrium Dissociation Constants for Cations Bound to PLA2. Values of dissociation constants for cations bound to PLA2 under different conditions were determined by the protection method. As shown in Figure 6A, the half-time for alkylation of His-48 changed appreciably in the presence of a divalent cation like calcium or cadmium and the effect was

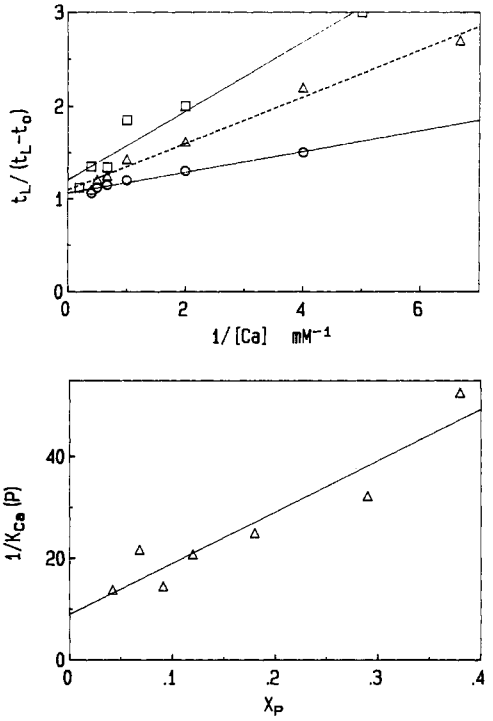

FIGURE 6: (A, top) Scrutton–Utter plot (eq 1) for the modification of PLA2 in (squares) the aqueous phase, (triangles) micelles of deoxy-LPC, and (circles) micelles of deoxy-LPC containing 0.0025 mole fraction MJ33. (B, bottom) $1/K_{Ca}$*(P) as a function of the mole fraction of products. When interpreted according to eq 2, such plots give values of K_P* (=0.03 mole fraction) and K_{Ca}* (=0.26 mM).

Table II: Equilibrium Dissociation Constants (mM) for Calcium and Ligand Bound to PLA2 at pH 7.3 and 23 °C

complex	K_{Ca}*	K_L^{Ca}*	k_L/k_0
EC	0.35		0.13
E*C	0.25		<0.07
E*CL (L = MJ33)	0.33	0.0009	<0.03

concentration dependent. The cation-dependent protection from alkylation was enhanced further in the presence of active-site-directed ligands; however, such a ligand alone in the absence of a cation did not protect the enzyme from alkylation. The values of the dissociation constants and k_L/k_0 for PLA2 in the presence of calcium obtained from the plots of the type shown in Figure 6 are summarized in Table II. The results show that, in the presence of calcium and a saturating concentration of active-site-directed ligand, His-48 was virtually completely shielded from alkylation ($k_L/k_0 < 0.03$ from eq 1). In the presence of calcium, but in the absence of an active-site-directed ligand, the k_L/k_0 was about 10% of that observed in the absence of the cation.

Values of K_C for the cation bound to the enzyme in the aqueous phase are summarized in Table I. Except for barium and strontium, for all other cations the values for K_C are up to several hundred-fold larger than the K_C*(S) values obtained from the inhibition kinetics. Such a behavior would be expected if ions like calcium and cadmium synergistically promoted the binding of the substrate to the active site. In fact, values of K_C*(S) calculated according to eq 4 and summarized in Table I are consistent with the experimentally measured values of K_{Ca}* and K_M*. The only major exception is observed in the kinetic results with barium and strontium for which the K_C and K_C*(S) values were approximately the same. This suggests that the inhibitory effect of these cations is manifested by a different mechanism, which can only be explained in terms of scheme III with the assumption that

Table III: Equilibrium Dissociation Constants (mM) for Ions Bound to E, E*, and E*DTPM Forms of Pig Pancreatic PLA2

constant	units	C = Ca^{2+}	C = Cd^{2+}	C = Ba^{2+}	C = Sr^{2+}
K_c(EC)	mM	0.35	0.16	0.5	0.16
K_c*(E*C)	mM	0.25	0.065	0.10	0.14
K_L*	mole fraction				
L = MG14		0.0011	0.0009	0.02	0.03
L = MJ33		0.0009	0.0075	>0.05	0.03
L = RM2		0.0023	0.003	<0.05	0.03
L = L-DTPM		0.03	0.017	0.09	
L = D-DMPM		0.04			
L = DTPC		0.06			
L = products of DMPM		0.03	0.044		
L = lyso-GPM		0.04			
L = myristic acid		0.3			
products of DMPC		0.065	0.1		
products of (S_p)-DPPsC		0.10	0.04		
products of (R_p)-DPPsC		0.125	0.05		

these cations bind to the enzyme but they do not support the binding of the substrate to the enzyme; i.e., K_{Ba}*(S) = K_{Ba}* = K_{Ba}. Therefore, according to eq 4, the kinetic IC(50) values obtained in the presence of calcium for barium and strontium would be larger than the corresponding K_C* value, as is indeed the case.

Obligatory Cation Requirement for the Binding of a Ligand. Active-site-directed ligands protect PLA2 from alkylation only in the presence of certain divalent cations; i.e., the complex of E* with a ligand is formed only in the presence of the cation. As shown in Figure 6B, values of the dissociation constant, K_L*, for the E*CL complex (at the saturating divalent cation concentration) were obtained from experiments designed on the basis of eqs 2 and 3. Values of K_L* obtained in the presence of the saturating divalent cation concentration are summarized in Table III.

Values of K_L* for the analogs of the substrate (L-DTPM and D-DMPM) were essentially the same as for the products of hydrolysis. Similarly, values of K_L* with saturating calcium (0.03–0.06 mole fraction) were only slightly larger than the values obtained with cadmium (0.017–0.05 mole fraction). These results suggest that the oxygen of the *sn*-2-carbonyl group contributes little toward the binding of the ligand to the enzyme. This conclusion was supported by K_P* values. For both the products of hydrolysis together K_P* is the same as that for the lysophospholipid alone, whereas fatty acid alone showed little protection from alkylation. Similarly, the K_P* values for the products of hydrolysis of the *sn*-3-thiophosphate substrate, (S_P)- and (R_P)-DPPsC, were only slightly larger for the R_P analog with both the cations, although the K_P* values with both the enantiomers were smaller with cadmium. These results would suggest that the discrimination between the S_P and R_P enantiomers of lysophospholipids is relatively small as expected if the *sn*-3-phosphate did not contribute markedly toward the stability of the E*L complex.

The binding of the *sn*-2 tetrahedral transition-state mimics, MJ33 and MG14, to PLA2 was observed only in the presence of cations. Both of these inhibitors contain a phosphate or a phosphonate group in the *sn*-2 position, whereas MG14 also has the *sn*-3-phosphodiester group. Results summarized in Table III show that, in the presence of calcium or cadmium, MJ33 is a specific competitive inhibitor of PLA2 with the same potency as MG14. Here again in the presence of calcium the affinity is somewhat lower than that in the presence of cadmium; however, in both cases the affinities are more than an order of magnitude higher than those observed for DTPM and the products of hydrolysis. Our attempts to discriminate

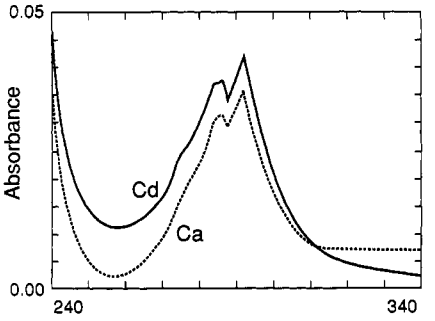

FIGURE 7: Changes in the UV absorbance spectrum induced in a mixture of PLA2 (0.028 mM), deoxy-LPC (1.2 mM), and MJ33 (0.1 mM) with 0.1 mM Cd or Ca at pH 8.0 in 20 mM Tris·HCl at 23 °C.

the coordination selectivity with enantiomeric thiophosphonate analogs of MG14 (Jain et al., 1989) by the protection method were unsuccessful because these analogs apparently reacted with the alkylating agent.

Calcium and cadmium support the binding of virtually all active-site-directed ligands to PLA2, and the K_L* values are comparable in the two cases. As summarized in Table III, barium and strontium do not effectively support the binding of the *sn*-2 transition-state analogs MJ33 and MG14, or of RM2, an *sn*-2-amide (Jain et al., 1991d). These results show that only calcium and cadmium accommodate the *sn*-2 tetrahedral transition state of glycerophospholipids.

In this paper we have not reported the binding of soluble monomeric active-site-directed ligands to PLA2. Under the conditions that we have examined only a weak binding of ligands can be seen in the presence of calcium (Rogers at al., 1992) and no binding in the absence of a divalent cation. These results are in apparent conflict with the observation of Volwerk et al. (1974) that a weak protection was observed in the presence of fatty acids and short-chain monoacyl- and diacylphosphatidylcholines at the monomeric concentrations in the absence of calcium. A possible explanation for this observation may be that such amphiphiles compete with the alkylating agent for the binding to the hydrophobic pocket leading to His-48.

Spectral Properties of PLA2 in the Presence of Cadmium. The fluorescence and absorbance properties of PLA2 change on binding to the interface, and the changes observed in the presence of cadmium are indistinguishable from those seen with calcium for the E, E*, and E*L forms of the enzyme. The difference absorbance spectra obtained on the binding of MJ33 to PLA2 on deoxy-LPC micelles in the presence of cadmium and calcium are shown in Figure 7. This suggests that the cation- and ligand-dependent perturbations are qualitatively similar. A small quantitative difference could be related to the sequential equilibria, E $\rightleftharpoons$ E* $\rightleftharpoons$ E*L, because the absorbance at 292 nm increased as a function of the mole fraction of the inhibitor and its apparent dissociation constant.

The fluorescence emission intensity of PLA2 at 335 nm also increased with the mole fraction of inhibitor (results not shown). The effective mole fraction of inhibitor required for a 50% change in the fluorescence signal is different than that observed for the change in the absorbance. This is probably because the concentration of PLA2 for these measurements is different, 2 versus 35 μM. If corrected for this fact, the values of K_I* for MJ33 obtained by the two methods are 0.000 55 and 0.001 mole fraction, which are in the range for the value obtained by the protection method.

The effect of cadmium on the binding of MJ33 to the active site of PLA2 bound to the micellar interface of deoxy-LPC

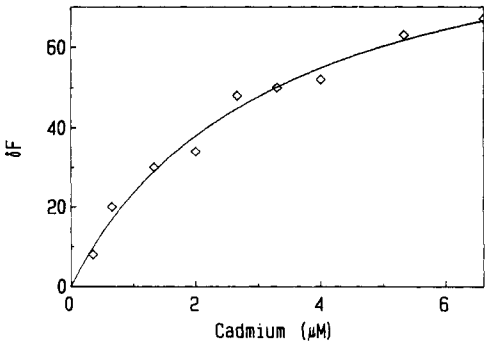

FIGURE 8: Change in the fluorescence emission intensity at 333 nm as a function of cadmium added to the reaction mixture containing PLA2 (0.004 mM), deoxy-LPC (3.3 mM), and 0.1 mM MJ33 at pH 8.0 in Tris·HCl at 23 °C.

as monitored by the fluorescence change is shown in Figure 8. The effective $K_C^*(I)$ for cadmium obtained from such plots is 0.0019 mM, which according to eq 2 and $K_I^* = 0.000\,75$ corresponds to $K_{Cd} = 0.065$ mM, compared to a value of 0.06 mM obtained by the protection method.

Finally it may be noted that the spectral changes characteristic for the formation of E* and E*L forms in the presence of calcium or cadmium were not observed in the presence of barium. This confirms the kinetic evidence that the E*L state is not formed in the presence of barium.

DISCUSSION

The effect of cations on interfacial catalysis by PLA2 can be adequately interpreted in terms of scheme III (Figure 1) which is an expanded version of the minimal kinetic scheme for interfacial catalysis (Jain & Berg, 1989; Berg et al., 1991) to accommodate the cation-dependent equilibria. Results show that *the divalent ion at the active site of PLA2 is obligatorily required for the synergistic binding of the active-site-directed ligands. This is consistent with the mechanism where the cation binds to the enzyme before the binding of the substrate.* This conclusion is at variance with the conclusions of de Haas and co-workers (1971) who suggested that the binding of the substrate and calcium follow a random pathway. In these early studies the binding of the enzyme to the interface or to the premicellar aggregate was not dissected from the steps of the catalytic turnover cycle. Any effect of calcium observed under the kinetic conditions used by these researchers would represent the overall effect on any one or more of the steps in the sequential $E \rightleftharpoons E^* \rightleftharpoons E^*S \rightarrow E^* + P$ equilibria. In fact, similar effects could also be responsible for the anomalous kinetic effects of calcium observed under other conditions. For example, the K_{Ca} values obtained under the scooting kinetic conditions (Berg et al., 1991; Jain et al., 1991c) are comparable to those predicted from the protection method; however, considerably larger values have been reported with micellar substrates (Verheij et al., 1982). We have not investigated such systems in detail; however, in micelles the rate of hydrolysis is limited by the depletion of the substrate (Jain et al., 1991c, 1992). Therefore, it is possible that under such conditions calcium plays an additional role of promoting fusion and fission of micelles to relieve the substrate depletion on the enzyme-containing micelles (Jain et al., 1992b).

Ions like cadmium and other alkali-metal cations bind to the calcium site in several proteins (Tsai et al., 1985; Villafranca et al., 1992). Our results show that not only does cadmium support the binding of active-site-directed ligands and hydrolysis of the substrate to PLA2, but the conformational state of the enzyme as judged from its spectroscopic properties is virtually the same as that of the calcium-containing enzyme. Thus, the cadmium-containing PLA2 should be useful for biophysical characterization of the complexes of the substrate and its analogs. Such studies should ultimately help in understanding the role of the cation in the catalytic process. Ions like barium have been used for the interpretation of results with the "suicide substrate" (Washburn & Dennis, 1991). In view of the fact that barium does not support binding of a ligand to the active site of PLA2, the mechanism proposed by these researchers for the putative suicide substrates is certainly doubtful.

The overall effect of cations as reported in this paper can be rationalized in terms of the following properties of the cations.

(1) The effect of charge is probably best reflected in the K_C values for the EC complexes in the aqueous phase. The values for the trivalent cations are lower by an order of magnitude. However, the charge difference does not show up in the $K_C^*(L)$ values obtained during the protection or the conditions for inhibition kinetics.

(2) Ionic radii are critical for the fit of a cation into the cavity of the protein. In the EC complex this difference is not reflected, probably because of the flexibility afforded by the two water molecules as ligands in the pentagonal bipyramidal geometry around the bound calcium. As water is replaced by oxygens of the substrate, the difference between different cations becomes more significant in the ES complex. For example, larger (like Ba or Sr) cations do not support the formation of E*CL. With smaller cations like magnesium, the desolvation tendency is probably not favored, and therefore they also do not support the formation of E*L.

(3) The symmetry considerations arise from the ligation number and the coordination geometry. In ions with spherical symmetry (like Ca and Cd) the size of ligands dictates the ligation number and geometry. Calcium and cadmium promote the binding of ligands that would not bind to the active site in the absence of the cation. In energetic terms about 1.5 kcal/mol of the change in the binding energy is provided by the interaction of the cation with the *sn*-3-phosphate.

(4) The tetrahedral transition-state mimics with an *sn*-2-phosphonate or an *sn*-2-phosphate group bind with an incremental free energy of about 4 kcal/mol, corresponding to the dissociation constant of 0.0008 mole fraction. Results with MJ33 suggest that a phosphodiester group at the *sn*-3 position does not contribute toward the stability of such complexes with the transition-state mimics. Therefore, for the coordination with the cation, the ligand(s) from the *sn*-2-phosphate could be energetically dominant.

Although the ligand binding to the catalytic site of PLA2 can be rationalized in terms of charge, size, and ligation, the basis for an additional role of calcium is yet to be established; i.e., *unlike all other ions reported here calcium supports catalysis.* This indicates that in the transition state not only the geometry of the active-site-directed ligand is important, but the coordination geometry of the cation in the transition state is somehow critical for the electrophilic behavior of the catalytic site. In other words, is it possible that the ternary ECL complex formed with cadmium has a different coordination number than that with calcium? We are investigating such possibilities which imply that with such cations the coordination geometry observed in the transition state could

be significantly different than the pentagonal bipyramidal geometry observed in the ground states of the E(Ca) and E(Ca)L complexes by X-ray crystallography.

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