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The Effect of Phospholipase A_2 Immobilization upon Calcium Interaction: A Kinetic Study[1]

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In this work we studied the effect of Ca^{2+} on the ability of immobilized PLA_2 to hydrolyze phospholipid substrates either in aggregate or monomeric forms. We use a kinetic methodology for the determination of dissociation constants of soluble and immobilized PLA_2-Ca^{2+} complexes. This approach allows us to obtain the values of the dissociation constants of enzyme-Ca^{2+} (K_x) and enzyme-Ca^{2+}-substrate (K'_x) complexes from the kinetic data obtained at different substrate and Ca^{2+} concentrations. Results using mixed micelles of phospholipid-Triton X-100 showed that, in most cases, productive complexes were destabilized by immobilization of PLA_2. However, a correct analysis of the interaction must be independent of the classical modes of PLA_2 action toward lipid surfaces. Thus, a substrate in monomeric form was also employed to analyze the effect of immobilization on hydrolysis rate in the absence of interfacial activation. Kinetic data showed that the immobilization affected severely the mode of PLA_2 action. The kinetic data also suggested that immobilization promoted conformational alterations in the Ca^{2+}-binding site, destabilizing the productive complex enzyme-Ca^{2+}-phospholipid.

Key words: calcium activation, immobilization, kinetic, phospholipase A_2, phospholipid hydrolysis.

In biological media, enzymes frequently act in naturally immobilized forms. The mode of immobilization can range from the relatively weak (primarily electrostatic) interactions characteristic of peripheral proteins, to the strong (predominantly hydrophobic) interactions of integral proteins (*1*). Bound enzymes rather than soluble or free forms could be the "*in vivo*" catalytic agents (*2*). Immobilized enzymes (IE) can display different properties from the respective soluble enzymes (SE) in solution. Alterations can be observed in pH optimum, binding constants, reaction rates, stability, and selectivity (*3*). Furthermore, the removal of an enzyme from its membrane environment can lead to changes in properties ranging from complete loss of activity to modifications in the kinetic mechanism (*4*).

A more realistic compromise between classical enzymology and the complex biological systems could be reached by studying enzymes in immobilized forms. Phospholipases develop their catalytic action at interfaces, and the interfacial activation phenomenon in the phospholipid hydrolysis is well known (*5*). For example, phospholipase A_2 (PLA_2) [EC 3.1.1.4] acts either in the scooting (*6*) or in the hopping mode of hydrolysis (*7*), depending on the enzyme source and the substrate type. In either case, the catalyst is activated at the phospholipidic surface, then develops its action by promoting the release of a fatty acid and a lysoderivative molecule. This mechanism may not fit well for an immobilized PLA_2. Furthermore, considering the essential activator (*8*) role of Ca^{2+} in the mechanism of action of secretory PLA_2s, the study of immobilization effects on binding constants specifically referred to as immobilized PLA_2-Ca^{2+} interaction is of great importance. PLA_2 has received considerable attention due to its involvement in a variety of different physiological and pathological functions (*9*).

The study of the binding of soluble ligands to surfaces could be complex. Most of the methods applied to interaction studies (spectroscopic and others) are not completely useful here, because the ligand is interacting with an insoluble bound enzyme. Titration microcalorimetric methodology may be suitable for this purpose, but the heat involved in the interaction can be masked by lipid hydrolysis. Nevertheless, only kinetic methods offer information about specific interactions involved in the catalysis. In this paper we use a kinetic method to obtain dissociation constants of immobilized PLA_2-Ca^{2+} complexes. This attempt is based on the interaction theory model for enzyme activation proposed by Segel (*8*) and Cornish-Bowden (*10*). In this report we have compared the behavior of PLA_2 in its soluble and in immobilized forms towards micellar and monomeric substrates, in order to investigate

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Abbreviations: SE, soluble enzyme; IE, immobilized enzyme; CM-Sephadex, carboxymethyl Sephadex; Tris, Tris(hydroxymethyl) aminomethane; PC, phosphatidylcholine; PLA_2 phospholipase A_2; SL, soybean lecithin; CMC, critical micellar concentration.

the influence of the topological state of the substrate on the immobilized system, especially on the enzyme-Ca^{2+} interaction. By comparing the dissociation constant values of calcium complexes and the kinetic parameters of both phospholipase forms, it is possible to analyze different immobilization effects.

MATERIALS AND METHODS

Reagents and Chemicals—PLA_2 from bee venom (*Apis mellifera*) was purchased from Sigma Chem. (St. Louis, USA) and used without further purification. The enzyme has an apparent molecular mass of 19 kDa and an isoelectric point of 10.5 (*11*). Stock and assay solutions of enzyme were prepared in 50 mM Tris-HCl, pH 8.2. PLA_2 from cobra venom (*Naja naja naja*) in phosphate buffer saline (PBS) solution 0.35 $\mu g/\mu l$ was also used. The *N. naja naja* enzyme was a gift from Dr. I. Bianco. This PLA_2 was purified as described in Ref. *12*.

Carboxymethyl Sephadex C-25 (CM-Sephadex), a weakly acidic cation exchanger, was obtained from Sigma. This polyanionic support is based on a crosslinked dextran matrix with a dry bead size of 40-125 μm and a capacity of 4.0-5.0 meq/g. Eupergit C, an uncharged support, was obtained from Pharma Polymers Rohm (Germany). This hydrophilic and electroneutral acrylic polymer contains oxirane derivatives as reactive groups that bind covalently sulfhydryl, amino, or hydroxyl compounds. The content of oxirane groups is higher than 600 μmol per gram (dry) (*13*).

Soybean lecithin (SL), a mixture of neutral and anionic phospholipids, was purified as described in Ref. *14*. Its composition was: phosphatidylcholine (33.4%), phosphatidylethanolamine (28.6%), phosphatidylinositol (29.6%), and phosphatidic acid (8.3%). The mixture was decationized to eliminate naturally present calcium. For this purpose, all the reactives were separately treated using a cation exchanger Dowex 50W×8 Rohm & Haas (Germany). Egg phosphatidylcholine (egg PC) was purchased from Sigma. Synthetic dihexanoyl phosphatidylcholine (Di-C6-PC), also from Sigma, was used as short-chain phospholipid substrate for kinetic studies in the monomeric state. All the phospholipids used were checked for calcium by absorption spectroscopy. Triton X-100 was obtained from Rohm & Haas. Tris, PBS, potassium barbital, calcium chloride, and all the other reagents were of analytical grade.

Enzyme Immobilization—Ionic immobilization of bee venom PLA_2 (*Apis mellifera*) onto CM-Sephadex was carried out according to a preceding paper (*14*). For the covalent coupling of cobra venom PLA_2 (*N. naja naja*), the enzyme solution in PBS was incubated for 24 h in the presence of the acrylic polymer Eupergit C. The immobilized PLA_2 system was filtered in vacuum to obtain the wet form of the immobilized enzyme (IE). Fixed activity was estimated from the activity of the initial enzyme solution and the remaining activity in the supernatant immediately after immobilization.

Enzymatic Assay—For long-chain phospholipids (SL and egg PC), the activity of either soluble enzyme (SE) or the immobilized form (IE) was estimated using phospholipid-Triton X-100 (molar ratio 1:4) mixed micelles. In the IE system the amount of immobilized PLA_2 was identical to that used in the SE assays. Assays were carried out in 50 mM Tris, pH 8.1, at 40°C in a defined range of $CaCl_2$. When the support of the immobilized system was Eupergit C, it was necessary to use a potassium barbital buffer instead of Tris, because of the reactivity toward oxirane groups of the latter. Short chain phospholipid (Di-C6-PC) solutions were prepared in Tris buffer with the corresponding amount of $CaCl_2$.

The reaction medium (2 ml) was rotated in a rotator system Buchi RE-111 for 1 min at 60 rpm. The control assay was performed without enzyme. PLA_2 activity (initial rate) was obtained by titrimetric microdetermination of released fatty acids as described previously (*14*). All the values given are averages of at least duplicate assays.

Determination of CMC of Di-C6-PC by ANS Fluorescence—The critical micellar concentration (CMC) value of the short-chain phospholipid Di-C6-PC was checked by fluorescence spectroscopy using 1-anilino 8-naphthalene sulfonic acid (ANS) as fluorescent probe. When the phospholipids aggregate, the relative fluorescence intensity increases abruptly. Fluorescence was excited at 350 nm and emission was measured at 490 nm. Dilutions were made in a concentration range below and above the reported CMC value of Di-C6-PC of 14 mM (*15*). The experimental value obtained was 12.5 mM.

Cornish-Bowden Treatment and Determination of Kinetic Parameters—The Cornish-Bowden treatment allows us to obtain the values of dissociation constants of enzyme complexes with Ca^{2+} activator. Essentially the same methodology was used to investigate the type of activation as has been used in linear inhibition (*16*), by replacing i (inhibitor) by $1/x$ and K_i (or K'_i) by $1/K_x$ (or $1/K'_x$), where x represents the activator concentration. For example, K_x was determined by a plot analogous to a Dixon plot in which $1/v$ is plotted against $1/Ca^{2+}$ at two or more values of phospholipid concentration (s); the abscissa corresponding to the interception point of the resulting straight lines gives $1/K_x$. A similar procedure but with s/v as the ordinate was followed to obtain K'_x. At the interception of the inverses, the velocities plotted at 5 and 10 mM substrate equalize, a fact on which the mathematical treatment to obtain K_x and K'_x is based (*10*). These constants and the enzymatic kinetic parameters (V_{max} and K_m) were determined using the GraFit software (*17*) by applying a nonlinear regression treatment for curve fitting. Results are the means of two or three independent experiments performed in duplicate.

Definition of Constants—Kinetic constants were derived from experimental data obtained at initial rate conditions, assuming a steady-state approach in which the complex concentration is a constant.

$K_{mCa^{2+}}$ = affinity apparent constant, refers to the amount of Ca^{2+} required to achieve one-half of the maximum initial rate achieved at 5 mM phospholipid concentration (provides general information on Ca^{2+} binding). K_x = equilibrium dissociation constant for the binary complex PLA_2-Ca^{2+} (provides specific information on Ca^{2+} binding). K'_x = equilibrium dissociation constant for the ternary complex PLA_2-Ca^{2+}-substrate (provides information on specific Ca^{2+} binding). V_{max} = maximum velocity. K_m = the usual Michaelis-Menten constant.

RESULTS

Immobilization—Bee venom PLA_2 (*Apis mellifera*), having positive net charge under the immobilization conditions, was fixed to CM-Sephadex by ionic adsorption. The non-covalent binding yielded a highly stable system and a considerable retention of enzymatic activity. The results were similar to those previously reported (*14*).

Covalent immobilization of cobra venom PLA_2 (*N. naja naja*) on the neutral acrylic polymer Eupergit C through oxirane groups was also satisfactory. The retained activity for this particular system was about 48% compared to the original soluble activity. Basically, this covalent linkage is the result of a nucleophilic attack on oxirane carbon. However, enzymes from such sources as bee venom or bovine pancreas lead to practically inactive immobilized adducts. Presumably, in these cases, the amine groups of the catalytic site are involved in the covalent binding and the enzyme loses most of its activity. In the case of *N. naja naja* PLA_2, covalent coupling onto acrylic beads yielded a stable IE: the conjugate maintained almost all the original activity through ten cycles of reaction with different batches of substrate.

Enzyme Activation Model in the Kinetic Assays Applied—There are direct methods to determine the equilibrium constants for ligands. Furthermore, in a related work (*18*), the dissociation constant values were obtained by monitoring the susceptibility to alkylation of His-48 at the catalytic site of pig pancreatic PLA_2. The same authors studied the role of Ca^{2+} binding in the stabilization of the transition state. Their results provided evidence for the hypothesis that the catalytic and the interfacial binding steps are kinetically and functionally distinct. Nevertheless, in the case of enzymes, only kinetic methods allow measurement of the effectiveness of the catalyst-ligand interaction, because the values of the constants are obtained directly from enzyme kinetic data.

In this work, the experimental design was based on four assay systems (A, B, C, and D) for soluble (SE) and immobilized phospholipase A_2 (IE), as described in Table I. Table I reports the kinetic parameters V_{max} and K_m obtained by non-linear regression fitting of the data (*17*) according to similar experimental conditions as described previously by the authors (*14*). Figures 1 to 4 show the curves representing initial rates against Ca^{2+} concentration for SE and IE for all the systems assayed at 5 and 10 mM substrate. Table II show the values of Ca^{2+} dissociation constants obtained from the respective inverse plots, as described in "MATERIALS AND METHODS." In system D, in which the short-chain Di-C6-PC substrate has a CMC of

TABLE I. **Kinetic parameters for soluble and immobilized PLA_2s in the different systems.**

System	Parameter	Soluble enzyme (SE)	Immobilized enzyme (IE)	SE to IE parameter ratio
		(Normalized values[a])		
A	K_m	1	0.47	2.1
	V_{max}	100	54	1.9
B	K_m	1	0.31	3.2
	V_{max}	100	35	2.9
C	K_m	1	0.92	1.1
	V_{max}	100	51	2.0
D	Initial rate at 10 mM Di-C6-PC	150±5 (U/mg)	136±6 (U/mg)	1.1

[a]The values were normalized relative to V_{max} (100 U/mg) and K_m (1 mM) obtained for soluble enzymes (SE). However, SE and IE in each of systems A, B, and C were prepared with the same batch of enzymes. In systems A, B, and D, the enzyme used was bee venom PLA_2 (*Apis mellifera*), and the immobilized form was CM-Sephadex-PLA_2. In system C, the enzyme was cobra venom PLA_2 (*Naja naja naja*) and the covalent immobilized form was Eupergit-PLA_2. The amount of PLA_2 in all systems was 10 μg in a final reaction volume of 2 ml. For system D, the values of the initial rate at the maximal concentration of substrate that can be achieved in the monomeric form (di-C6-PC) are reported. The substrate in systems A, B, and C was phospholipid (respectively egg PC, SL, and egg PC) in mixed micelles with Triton X-100 (1:4 molar ratio) in a concentration range of 2 to 30 mM. The Ca^{2+} concentration for all the systems was near to that of the maximal activation effect of the cation (at least 0.5 mM).

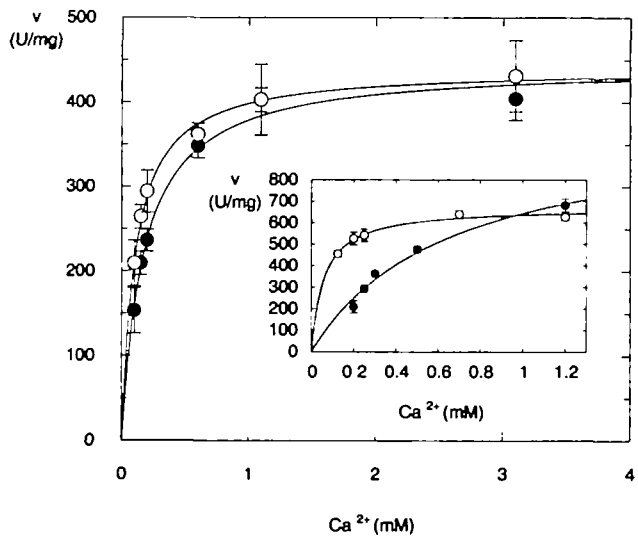

Fig. 1. **Effect of calcium concentration on egg PC (mixed micelles) hydrolysis by soluble and immobilized PLA_2s from bee venom (system A).** The initial rate was measured at 5 mM (main figure) or 10 mM (insert) of substrate for soluble (○) or immobilized on CM-Sephadex (●) PLA_2. See "MATERIALS AND METHODS" for details. Bars represent mean±SDM (standard deviation mean).

TABLE II. **Dissociation constants of Ca^{2+} complexes and K_{mCa} values for soluble and immobilized PLA_2s in all the systems assayed.**

Parameter	System A		System B		System C		System D	
	SE	IE	SE	IE	SE	IE	SE	IE
$K_{m\ Ca}$	0.11±0.01	0.18±0.02	0.05±0.01	0.14±0.02	0.14±0.02	0.32±0.04	0.90±0.14	0.76±0.09
K_x	0.20±0.02	0.21±0.03	0.05±0.01	0.08±0.02	0.21±0.01	0.08±0.02	2.10±0.20	0.75±0.11
K'_x	0.10±0.01	1.18±0.16	≅0.001	0.04±0.01	5±1	6±1	0.98±0.08	3.85±0.32

Systems A, B, C, and D were defined in Table I. The values of constants are the means of two or three independent experiments, ±SDM (standard deviation mean). See "MATERIALS AND METHODS" for experimental details.

12.5 mM, the kinetic constants (V_{max} and K_m) could not be calculated, since a complete Michaelian curve could not be achieved at substrate concentrations below the CMC (the monomeric region). Similar behavior was reported by other authors (*19*). However, PLA_2 activities at 5 and 10 mM Di-C6-PC allowed us to compare SE and IE. Both the SE and the IE gave similar initial rates *vs.* Ca^{2+} profiles at high substrate concentration.

In this report, we mainly studied the influence of PLA_2 immobilization upon Ca^{2+}-enzyme interaction and its kinetic implications. The analysis was based on the general model of activation described previously (*8, 10*). Basically, the model contemplates three kinds of mechanisms: (i) specific activation, (ii) mixed activation, and (iii) partial or non-essential activation. The PLA_2 activity was null in the absence of Ca^{2+}, and this fact is consistent with an essential activation mechanism. Hence, the general scheme is:

$$\begin{array}{ccccc} S+EX & \rightleftharpoons & EXS & \rightleftharpoons & EX+P \\ \Big\uparrow\Big\downarrow K_x & & \Big\uparrow\Big\downarrow K'_x & & \\ E+X & & ES+X & & \end{array}$$

Where: S is the substrate (phospholipid), E is the PLA_2 enzyme (either SE or IE form), and X is the activator (Ca^{2+}). K_x and K'_x are the dissociation constants of the enzyme complexes EX and EXS, respectively. The corresponding velocity equation is given by Ref. *10*:

$$v=\frac{V\cdot S}{K_m(1+\frac{K_x}{X})+S(1+\frac{K'_x}{X})}$$

where V and K_m are the limiting rate and Michaelis constant respectively for the activated enzyme, *i.e.,* PLA_2 with Ca^{2+} bound to it.

Kinetic Assays Using Micellar Substrates—In system A, IE showed a greater affinity (minor K_m) toward egg PC than the soluble form (SE) (see Table I). The large surface of the support may offer additional non-specific sites of substrate, thereby improving the contact with the enzyme (*20*). However, the IE had a lower activity with a V_{max} about two times lower than SE, indicating a less effective catalytic cycle. Two reasons can be considered to explain this behavior. First, the immobilized PLA_2 does not achieve a complete interfacial activation; and second, although the substrate-binding site was not adversely affected, the immobilization takes place with changes in the conformation of the catalytic domain involving His 48 and Asp 99 (*21*) or, alternatively, the support imposes some physical constraint on the tertiary structure of the protein. In most naturally occurring biological membranes the net charge is negative, and it has been shown that the density of surface charge could alter the behavior of several enzymes located at the membranes (*22*). In system A, PLA_2 ionically immobilized onto CM-Sephadex and acting upon egg PC/Triton mixed micelles showed in general a lower Ca^{2+} affinity (higher K_{mCa}, see Table II). The activator requirement was greater for the IE form, indicating that the polyanionic support competes directly with the enzyme in Ca^{2+} binding, or that some conformational change alters the cofactor interaction domain of the enzyme. Two glycine residues and one aspartic acid (Asp 49) in PLA_2 seem to be involved in the Ca^{2+}-binding domain (*21*). The experimental data obtained with system A suggest that immobilization does not affect the specific interaction PLA_2-activator, since similar values of K_x were obtained for soluble and immobilized form (Table II). However, the dissociation constant of the ternary productive complex PLA_2-Ca^{2+}-phospholipid (K'_x) for the IE was 12 times higher than SE. This greater instability suggests that conformational changes might alter the specific site of the interaction enzyme-substrate-activator. The same represents the proper interaction with enzymes, and hence the large difference between the two PLA_2 forms is not related to Ca^{2+}-solid support binding. In principle, K'_x is a dissociation constant for specific activator binding not including other hypothetical interactions. Since the Tris buffer concentration was at least 10-fold higher than the cofactor concentration, a clear difference in this equilibrium constant probably reflects a direct effect due to the immobilization and not an effect of the support by sequestering Ca^{2+}. Ionic interactions will be negligible in solutions of high ionic strength. Higher K'_x value for IE indicates lower specific affinity for the activator (EXS more dissociated). As a consequence, the activation effect was lower for IE at low calcium concentration, but the differences between the bound and soluble forms of the enzyme tend to disappear as Ca^{2+} concentration increases (Fig. 1). Mechanistically, in accordance with the Cornish-Bowden approach (*10*), our results were consistent with a mixed activation model. In this case, the activator is not indispensable for substrate binding, but only for catalysis. This suggests a random mechanism in which Ca^{2+} combines with PLA_2 independently of phospholipid and the latter combines with the enzyme independently of the activator. Nevertheless, only a ternary complex (EXS) leads to products.

The reduction of V_{max} upon enzyme immobilization in system B was in keeping with the values obtained for system A but more pronounced (see Table I). We also included substrate with negatively charged phospholipid (SL). In this case, immobilization led to a lower affinity for Ca^{2+} (IE had higher K_{mCa}), but the effect was stronger than in the previous case (Fig. 2 and Table II). This could be interpreted as follows. It was described previously that the specific affinity of Ca^{2+} for phospholipids, including those negatively charged, was low, with dissociation constants in the 0.1 M order (*23*). However, the local calcium concentration at the negatively micelle surface should be higher due to the double layer effect, and the actual free bulk Ca^{2+} concentration that senses the immobilized enzyme is different (lower) than that of completely neutral substrate (system A). As the specific affinity of Ca^{2+} for the enzyme, soluble or immobilized, is in the mM range (Table II) and these values are about 10^2-10^3 times higher than the specific affinity of this cation for phospholipids (*23*), it can be concluded that the specific complex phospholipid-Ca^{2+} is not relevant in the proposed model of activation (see the scheme above). In this connection, similar conclusions were obtained earlier by de Haas and coworkers using pancreatic PLA_2 (*24*), in which the kinetic data were not in agreement with the viability of Ca^{2+}-phospholipid complex. The same conclusion was reached by Wells (*25*) using *Crotalus adamanteus* PLA_2 and the soluble dibutyrylphosphatidylcholine substrate. The immobilization in system B virtually modifies the mechanism, because K'_x was ~0 for SE. This indicates that the soluble enzyme interacts with the substrate only in its enzyme-activator complex state. In practice, from the Cornish-Bowden viewpoint, this situa-

tion configures either a specific type of activation model, or represents an extreme case of a mixed type activation model. On the other hand, the value of K'_x for IE was 0.04, indicating that the immobilized PLA_2 form without activator binds substrate with a higher affinity than the soluble form. For either SE or IE, the presence of anionic phospholipids (SL) leads to more stable ternary productive complexes (compared to pure zwitterionic PC). For system B, the negative surface charged substrate in an aggregate form plays an important role in the PLA_2-Ca^{2+} interaction, a property that has been explained in terms of a specific bridge of the metal between PLA_2 and the anionic phosphate group of the substrate (*26, 27*).

Although the optimum pH activity was similar for the soluble and immobilized PLA_2 forms when the support was CM-Sephadex (*14*), in the case of membrane-bound enzymes, interaction with ligands could be modified due to changes of the microenvironment close to biological membranes (*22*). For this reason, and to avoid non-specific ligand interactions, we designed an experiment using an immobilized PLA_2 bound to an electroneutral polymer (system C). In this third assay system, PLA_2 from cobra venom (*N. naja naja*) was covalently immobilized on an electroneutral polymer. This immobilized enzyme showed a lower affinity for Ca^{2+}, with a K_{mCa} for IE clearly higher than the respective value found for ES (Fig. 3 and Table II). In this system, no ionic interaction is possible between the activator Ca^{2+} and the support. Thus, structural alteration due to the covalent immobilization is the only factor responsible for the lower Ca^{2+} affinity observed for the IE system. Covalent protein coupling probably produces certain conformational changes in the Ca^{2+} binding domain that retains the cation (smaller K_x for IE). The values of K'_x, in both cases (ES and IE) were similar but much higher than those found in most of the other systems (compare Tables III, IV, and V). This seems to be an intrinsic characteristic of the *N. naja naja* enzyme.

The IE acting upon micellar substrate (systems A, B, and C) could mimic a typical biological situation in which naturally immobilized PLA_2 develops lipolytic action towards substrate inserted into biomembranes. Also, two action modes had been proposed in the phospholipid hydrolysis by mitochondrial PLA_2: the intramembrane mechanism and the intermembrane or interorganelle mechanism (*28*). In principle, this reasoning is consistent with the theory of interfacial recognition suggested by Ferreira *et al.* (*19*) to explain the action of an immobilized PLA_2.

Kinetic Assays Using Monomeric Substrate—For system D, the substrate was in the monomeric form and it was not possible to achieve a complete Michaelis curve to obtain V_{max} and K_m. However, it is noteworthy that at the highest monomeric concentration (10 mM) of substrate assayed, the rates of hydrolysis were similar for SE and IE independently of Ca^{2+} concentration (Fig. 4). A similar result was obtained by Lombardo and Dennis using cobra venom PLA_2 and diheptanoyl-PC (*29*). These results contrast with those obtained for micellar systems, in which the V_{max} for SE was always higher than that for IE. Ionic immobilization of the bee venom enzyme upon CM-Sephadex affected notoriously the specific domain of PLA_2-Ca^{2+}-Di-C6-PC interaction, since the dissociation constant of the ternary productive complex (K'_x) obtained for IE was four times higher than SE (Table II). This higher value of K'_x indicates that the connection of the protein to the support alters the Ca^{2+} binding in the EXS conformation. We observed differences in the behavior of the two PLA_2 forms only at the lowest substrate concentration (see Fig. 4). Differences in K'_x could not explain this effect, because the reaction rate values remained different even when calcium concentration

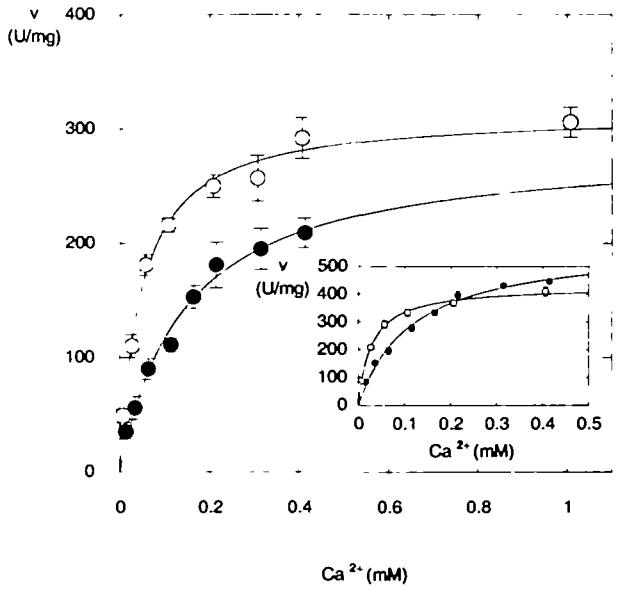

Fig. 2. **Effect of calcium concentration on soybean lecithin (SL mixed micelles) hydrolysis by soluble and immobilized PLA_2s from bee venom (system B).** The initial rate was measured at 5 mM (main figure) or 10 mM (insert) of substrate for soluble (○) or immobilized on CM-Sephadex (●) PLA_2. See "MATERIALS AND METHODS" for details. Bars represent mean ± SDM (standard deviation mean).

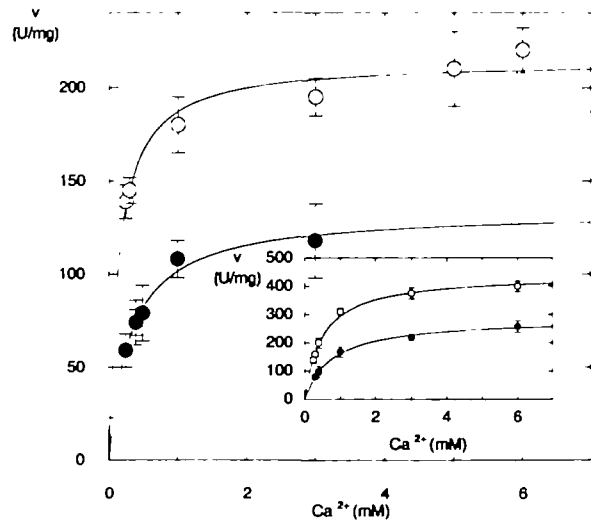

Fig. 3. **Effect of calcium concentration on egg PC (mixed micelles) hydrolysis by soluble and covalent immobilized PLA_2s from cobra venom (system C).** The initial rate was measured at 5 mM (main figure) or 10 mM (insert) of substrate for soluble (○) or covalent immobilized on Eupergit (●) PLA_2. See "MATERIALS AND METHODS" for details. Bars represent mean ± SDM (standard deviation mean).

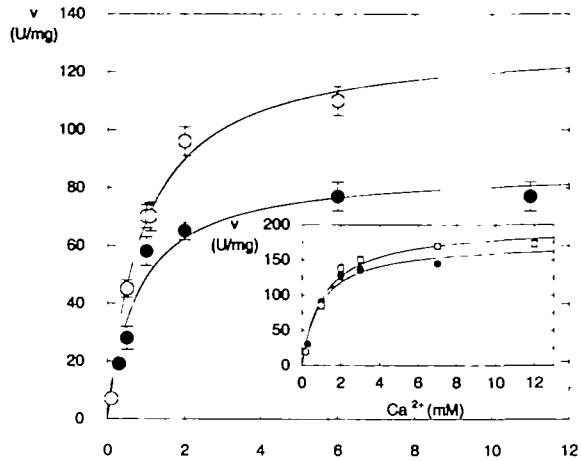

Fig. 4. **Effect of calcium concentration on Di-C6-PC (monomers) hydrolysis by soluble and immobilized PLA_2s from bee venom (system D).** The initial rate was measured at 5 mM (main figure) or 10 mM (insert) of monomeric substrate for soluble (○) or immobilized on CM-Sephadex (●) PLA_2. See "MATERIALS AND METHODS" for details. Bars represent mean±SDM (standard deviation mean).

was increased. A possible explanation is that 5 mM Di-C6-PC can not saturate the IE to form EXS productive complex. Immobilized PLA_2 probably remains in an unfavorable spatial position that hinders substrate binding.

For micellar systems A and B, immobilized PLA_2 tends to show a higher substrate affinity (lower K_m), presumably due to a facilitated targeting allowed by a favorable non-specific substrate-support interaction (*20*). However, the immobilization markedly decreases the value of V_{max} (see Table I). Two hypotheses were proposed above to explain the results obtained for system A. The results obtained with monomeric substrate (System D) validate the first hypothesis, in which the IE activity toward micelles is lower due to severe restrictions against operation in either the scooting or the hopping mode of interfacial hydrolysis. In these interfacial mechanisms the non-immobilized PLA_2 is free to diffuse on the surface of the aggregate substrate (*6*) or to hop among the micelles (*7*), respectively. With the monomeric substrate, neither the IE nor the SE can act in either of these mechanisms, and this explains why we did not find significative differences in enzyme activity at the highest monomeric substrate concentration possible (see Fig. 4 and Table II). In that condition, without a lipid surface, the behavior of both enzymatic forms was similar. These results also suggest that immobilization does not alter the catalytic step (both PLA_2 forms developed similar activity at 10 mM monomeric substrate), indicating that there is no substantial conformational change at the catalytic domain involving His 48 and Asp 99 (*30*). Mechanistically, system D configured a mixed activation model in which free or immobilized PLA_2 without calcium binds monomeric phospholipid (Di-C6-PC).

DISCUSSION

In micellar systems, the catalytic step was affected but essentially by altering the mode of PLA_2 action. A strong interaction with the solid support (*14*) would impose severe restrictions on the enzyme to work in the scooting or hopping mode. Taking into account the micelle-to-enzyme mole ratio (about 1,000 to 1) in our assays and the extent of hydrolysis (about 60% of the total substrate in 5 min, data not shown), it may be accepted that the SE enzyme works in the hopping mode (*7*). Also, it should be emphasized that the rate of hydrolysis was sensitive to bulk substrate concentration, contrary to the expected behavior if the enzyme works in the scooting mode (*6*). In principle, the lower V_{max} for IE in micellar systems it cannot be attributable to a loss of activity due to a random orientation of the PLA_2 adsorbed on the support, in which a certain amount of protein may be inadequately exposed (non active state), since similar activities of bound and soluble enzyme forms are found against higher monomeric substrate concentration. However, others factors may affect the behavior of immobilized enzyme, such as changes in the microenvironment, partitioning and mass transfer effect. Considering our experimental conditions and the observed IE properties (*14*), these factors would not be strongly involved in the differences in the kinetic parameters found for IE compared with SE. These factors were also previously analyzed by other authors, who reached a similar conclusion (*29*).

When the phospholipid was in monomeric state, the K_{mCa}s of both enzyme forms were several times higher than the respective values for micellar substrates. This suggests that the hydrophobic/hydrophilic character of the interface plays an important role in improving the steady-state binding of calcium to PLA_2. This is in agreement with previous studies (*31*).

Straight lines for reciprocals plots obtained from the data of Figs. 1 to 4 confirm that Ca^{2+} is an essential activator. Hence, the mixed activation mechanism (*10*) is in agreement with what is known about secretory PLA_2 catalysis. For these enzymes, Ca^{2+} seems not to be indispensable for substrate binding but only for catalysis. On the other hand, if hyperbolic plots instead of straight lines had been obtained for reciprocals, they would have indicated a non-essential (or partial) activator role for Ca^{2+}, which is not the case for secretory PLA_2s.

The interaction PLA_2-Ca^{2+}-phospholipid was dependent on the enzyme (SE or IE) form. In all systems assayed, we found that immobilization enhanced the dissociation of the ternary productive complexes, suggesting that immobilization promotes some conformational alterations in the specific domain of the interaction PLA_2-Ca^{2+}-phospholipid.

All IE analyzed for immobilization effects clearly satisfy a mixed mechanism type of activation (*10*). By this attempt we evaluated the interaction between the PLA_2 (soluble or immobilized) and its essential Ca^{2+} cofactor in the binary (PLA_2-Ca^{2+}) and the tertiary productive (PLA_2-Ca^{2+}-phospholipid) complexes.

REFERENCES

1. Price, N. (1995) Techniques for enzyme extraction in *Enzyme Assays. A Practical Approach* (Eisenthal, R. and Danson M., eds.) pp. 261-262, IRL Press, Oxford
2. Sackman, E. (1996) Supported membranes: scientific and practical applications. *Science* **271**, 43-48
3. Wolfbeis, O. (1993) Immobilized enzymes in optical biosensors in *Immobilized Macromolecules: Application Potentials* (Sleytr, U., Messner, P., Pum, D., and Sára, M., eds.) pp. 161-165, Springer-Verlag, London

4. Tipton, K. (1995) Principles of enzyme assay and kinetic studies in *Enzyme Assays. A Practical Approach* (Eisenthal, R. and Danson, M., eds.) pp. 47-49, IRL Press, Oxford
5. Scott, D., White, S., Otwinowski, Z., Yuan, W., Gelb, M., and Sigler, P. (1990) Interfacial catalysis: the mechanism of action of phospholipase A_2. *Science* **250**, 1541-1546
6. Jain, M. and Gelb, M. (1991) Phospholipase A_2-catalized hydrolysis of vesicles: Uses of interfacial catalysis in the scooting mode in *Methods in Enzymology, Phospholipases* (Dennis, E., ed.) Vol. 197, pp. 112-125, Academic Press, New York
7. Berg, O., Yu, B., Rogers, J., and Jain, M. (1991) Interfacial catalysis by phospholipase A_2: determination of the interfacial kinetic. *Biochemistry* **30**, 7283-7297
8. Segel, Y. (1993) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, John Wiley & Sons, New York
9. Dennis, E. (1994) Diversity of group types, regulation, and function of phospholipase A_2. *J. Biol. Chem.* **269**, 13057-13060
10. Cornish-Bowden, A. (1995) *Fundamentals of Enzyme Kinetics*, Portland Press, London
11. Shipolini, R., Callewaert, G., Cottrell, R., Doonan, S., Vernon, C., and Banks, B. (1971) Phospholipase A from bee venom. *Eur. J. Biochem.* **20**, 459-468
12. Reynolds, L. and Dennis, E. (1991) Cobra venom phospholipase A_2: *Naja naja naja* in *Methods in Enzymology, Phospholipases* (Dennis, E., ed.) Vol. 197, pp. 359-365, Academic Press, New York
13. Eupergit Basic Information—Information leaflets Pharma Polymers, Rohm, Germany
14. Madoery, R., González Gattone, C., and Fidelio, G. (1995) Bioconversion of phospholipids by immobilized phospholipase A_2. *J. Biotechnol.* **40**, 145-153
15. Roberts, M. (1991) Assays of phospholipases on short-chain phospholipids in *Methods in Enzymology, Phospholipases* (Dennis, E., ed.) Vol. 197, pp. 95-112, Academic Press, New York
16. Cornish-Bowden, A. (1974) A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem. J.* **137**, 143-144
17. Leatherbarrow, R. (1990) *GraFit Version 2.0*, Erithacus Software Ltd., Staines, UK
18. Jain, M., Yu, B., Rogers, J., Ranadive, G., and Berg, O. (1991) Interfacial catalysis by phospholipase A_2: dissociation constants for calcium, substrate, products and competitive inhibitors. *Biochemistry* **30**, 7306-7317
19. Ferreira, J., Sasisekharan, R., Louie, O., and Langer, R. (1993) Influence of chemistry in immobilization of cobra venom phospholipase A_2: implications as to mechanism. *Biochemistry* **32**, 8098-8102
20. von Hippel, P. and Berg, O. (1989) Facilitated target location in biological systems. *J. Biol. Chem.* **264**, 675-678
21. Kuchler, K., Gmachl, M., Sippl, M., and Kreil, G. (1989) Analysis of the DNA for phospholipase A_2 from honey bee venom glands. *Eur. J. Biochem.* **184**, 249-254
22. Wojtczak, L. and Nalecz, M. (1979) Surface charge of biological membranes as a possible regulator of membrane-bound enzymes. *Eur. J. Biochem.* **94**, 99-107
23. McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., and McLaughlin, A. (1981) Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* **77**, 445-473
24. De Haas, G., Bonsen, P., Pieterson, W., and van Deenen, L. (1971) Studies on phospholipase A in its zymogen from porcine pancreas. III Action of the enzymes on short chain lecithins. *Biochim. Biophys. Acta* **239**, 252-266
25. Wells, M. (1972) A kinetic studies of phospholipase A_2 (*Crotalus adamanteus*) catalyzed hydrolysis of 1,2 dibutyryl-*sn*-glycero-3-phosphorylcholine. *Biochemistry* **11**, 1030-1041
26. Volwerk, J., Jost, P., de Haas, G., and Griffith, O. (1986) Activation of porcine pancreatic phospholipase A_2 by the presence of negative charges at the lipid-water interface. *Biochemistry* **25**, 1726-1733
27. Scott, D., Mandel, A., Sigler, P., and Honig, B. (1994) The electrostatic basis for the interfacial binding of secretory phospholipases A_2. *Biophys. J.* **67**, 493-504
28. Winter, J., Lenting, H., Neys, F., and van den Bosch, H. (1987) Hydrolysis of membrane-associated phosphoglycerides by mitochondrial phospholipase A_2. *Biochim. Biophys. Acta* **917**, 169-177
29. Lombardo, D. and Dennis, E. (1985) Immobilized phospholipase A_2 from cobra venom. *J. Biol. Chem.* **260**, 16114-16121
30. Scott, D., Otwinowski, Z., Gelb, M., and Sigler, P. (1990) Crystal structure of bee venom phospholipase A_2 in a complex with a transition-state analogue. *Science* **250**, 1563-1566
31. Bent, E. and Bell, J. (1995) Quantification of the interactions among fatty acid, lysophosphatidylcholine, calcium, dimyristoylphosphatidylcholine vesicles and phospholipase A_2. *Biochim. Biophys. Acta* **1254**, 349-360