Zinc and barium inhibit the phospholipase A_2 from *Naja naja atra* by different mechanisms

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The mode of inhibition of the phospholipase A_2 (PLA_2) enzyme from the Chinese cobra (*Naja naja atra*) by Zn^{2+} is qualitatively different from inhibition by Ba^{2+}. Inhibition by Ba^{2+} shows the kinetic characteristics of a conventional competitive inhibitor acting to displace Ca^{2+} from a single essential site, but Zn^{2+} has the paradoxical property of being more inhibitory at high than at low Ca^{2+} concentration. Kinetic analysis of the Ca^{2+}-dependence of enzymic activity shows a bimodal response, indicating the presence of two Ca^{2+}-binding sites with affinities of 2.7 μM and 125 μM respectively, and we propose that these can be identified with the two Ca^{2+}-binding sites revealed by crystallographic analysis [White, Scott, Otwinowski, Gleb and Sigler (1990) Science **250**, 1560–1563]. The results are consistent with the model that the enzyme is activated by two Ca^{2+} ions, one that is essential and can be displaced by Ba^{2+}, and one that modulates the activity by a further 5–10-fold and which can be displaced by Zn^{2+}. An alternative model is also presented in which the modulating Zn^{2+}-binding site is a phenomenon of the lipid/water interface.

INTRODUCTION

Venom phospholipase A_2 (PLA_2) enzymes (EC 3.1.1.4) are Ca^{2+}-dependent enzymes, in which the Ca^{2+} ion contributes to the formation of the active site. Crystallographic analysis [1–4] has shown that the Ca^{2+} ion interacts with oxygen atoms belonging to an aspartate (Asp-49) and carbonyl groups of Trp-28, Gly-30 and Gly-32 residues in the enzyme and also to the phosphate oxygen in the substrate. Other bivalent cations can replace Ca^{2+}, and there is evidence that Sr^{2+} is a weak activator, whereas Ba^{2+} and Zn^{2+} are powerful inhibitors and a range of other ions are weak inhibitors [5,6]. Kinetic analyses support the model that Ca^{2+} binds to the enzyme before the substrate and predict that all antagonistic ions will affect the reaction kinetics in the same way, being functionally equivalent to decreasing the Ca^{2+} concentration [7]. Although there is evidence that Ba^{2+} binds to the same site as Ca^{2+} [8], there are no corresponding data for Zn^{2+}. It is, however, of considerable interest to know how a single site could bind ions of such disparate sizes [Ba^{2+}, Ca^{2+}, Zn^{2+}; 0.134 nm (1.34 Å), 0.099 nm (0.99 Å), 0.072 nm (0.72 Å) respectively], while having very low affinity for other transition metal ions. In the PLA_2 isoform from *Crotalus adamanteus*, Zn^{2+} and Ca^{2+} produce different spectral perturbations, indicating that they interact with different amino acid side chains [9,10], but this is not inconsistent with interaction at the same Ca^{2+}-binding site.

The aim of the present study was to analyse the metal-ion dependence of the PLA_2 enzyme from *Naja naja atra*. This enzyme was present in the venom as a single major isoform and was easy to purify in high yield. In addition, a full crystallographic analysis at 0.2 nm (2.0 Å) was available, which revealed that this enzyme, which was apparently a typical type-I PLA_2 enzyme according to the sequence data, possessed two Ca^{2+}-binding sites. This is not unique, because the PLA_2 enzyme from pig pancreas is known to bind a second Ca^{2+} ion with very low affinity and with a concomitant increase in substrate affinity at alkaline pH [11,12]. In this paper we present kinetic evidence that Ba^{2+} and Zn^{2+} inhibit venom PLA_2 enzymes by different mechanisms, and discuss the possibility that they act at different Ca^{2+}-binding sites.

MATERIALS AND METHODS

Materials

Naja naja atra venom was purchased from Sigma and the PLA_2 enzyme was purified by ion-exchange chromatography. Briefly, 200 mg of the whole venom was dissolved in 1 ml of distilled water in the presence of the protease inhibitor phenylmethanesulphonyl fluoride, and the pH was adjusted to 9.6 with ammonia solution. The sample was then applied to a column (2 cm × 5 cm) of Whatman DE-52 DEAE-cellulose equilibrated in 20 mM ammonium acetate, pH 9.6. When the initial run-through peak had been collected, the absorbed protein was eluted with 20 mM ammonium acetate to pH 5.0. The purity was established by acid/urea and alkaline/urea PAGE [13,14]. Glycerophosphocholine (GPC) was prepared from egg lecithin by a method designed to eliminate contamination by non-zwitterionic glycerophosphate derivatives. The purified lecithin, a mixture of phosphatidylcholine and phosphatidylethanolamine derivatives (~ 6:1, w/w), was subject to methanolysis, by using a macro-reticular resin (Amberlyst A26; OH^- form) as catalyst. GPC remained in the supernatant, but glycerophosphoethanolamine, which is anionic at high pH, was retained by the resin. The supernatant was recovered, dried, and the residue was extracted with ethyl acetate, dimethylformamide (to remove glycerol) and warm chloroform, and then extensively de-ionized in methanolic solution by using a mixed-bed resin (Dowex MR-3), until the conductance fell to that of pure methanol. Dioctanoylphosphatidylcholine (DOPC) was synthesized by a modification of the method of Patel et al. [15], with pyrrolidinopyridine catalyst, but the GPC free base was acylated directly. The derivatives were purified by chromatography on alumina, dried, redissolved in methanol, and then exhaustively

Abbreviations used: DOPC, dioctanoylphosphatidylcholine; GPC, glycerophosphocholine; NTA, nitrilotriacetic acid; PLA_2, phospholipase A_2 (EC 3.1.1.4).

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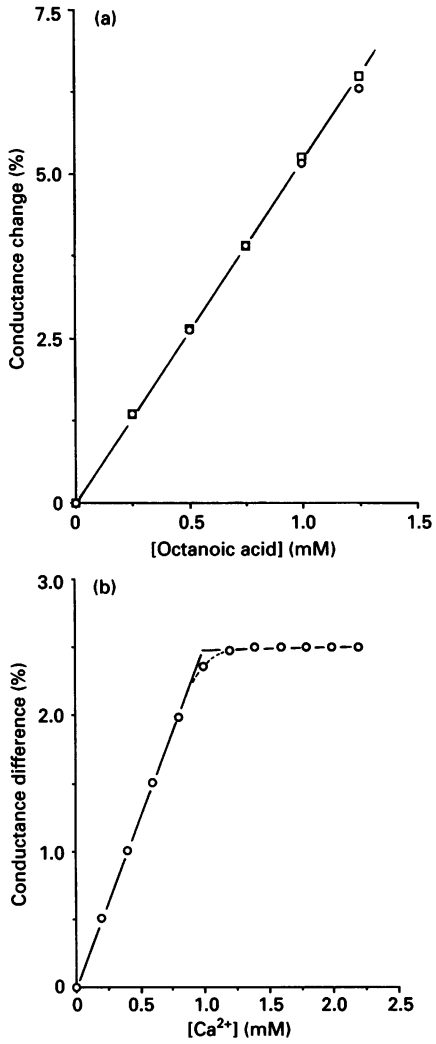

Figure 1 Assay calibration by conductimetric titration

(**a**) Calibration of the conductimetric assay method. Calibration was carried out by sequential injection of 2 μl portions of 500 mM octanoic acid solution in methanol into conductivity cells containing 2 ml of 10 mM triethanolamine/HCl buffer, pH 8.0, in the absence (□) and in the presence of 0.3 mM DOPC and 25 μM Zn^{2+} (○). The conductance values were corrected by subtraction of the effect of addition of 2 μl portions of pure methanol and plotted as a percentage of the total conductance change. Values are the means for three different determinations. (**b**) Calibration of the conductimetric assay method and calculation of free Ca^{2+}. The calibration assay was carried out by injecting 4 μl portions of 100 mM $CaCl_2$ into conductivity cells containing 2 ml of 10 mM triethanolamine/HCl buffer, pH 8.0, in the absence and presence of 1 mM NTA. Results were plotted as a difference curve for the absence and presence of NTA, which therefore represents the concentration of bound Ca^{2+} and enables the K_d to be determined from the relationship $K_d = [Ca^{2+}][NTA]/[Ca^{2+}\cdot NTA]$. The K_d value obtained from these data is 8 ± 1 μM.

deionized with Dowex MR-3 mixed-bed resin. The products gave single phosphomolybdate-positive, ninhydrin-negative, bands on t.l.c. Differential conductimetric titration of assay buffer with and without 0.3 mM phosphatidylcholine derivatives confirmed that contamination with bivalent metal cations was negligible.

PLA_2 assay

Enzyme assays were by conductimetry using methods described elsewhere [16–19], but with a significantly modified apparatus that used a set of eight 2 ml reaction cells sampled in sequence at 1 s intervals (Mezna and Lawrence [20]). Data could be recorded from any combination of cells at a maximum sampling rate of 1 s per point per cell. The conductance data were converted into 13-bit digital form (8196 divisions), corresponding to a total conductance change of $\sim 5\%$. Reaction rates were measured by a line-drawing program in which tangents to curves were drawn on the screen by using a pointer-controlled dragging procedure. One cell was reserved as a blank that could be subtracted from all other cells before further processing. Reactions were carried out at 37 °C in 10 mM triethanolamine buffer prepared by titrating 10 mM HCl to pH 8.0 with triethanolamine free base. All solutions were prepared from water purified by reverse osmosis, followed by two deionizing steps.

Calibration

Assay solutions were calibrated for linearity by conductimetric titration of the buffer by sequential additions of 2 μl portions of a 500 mM solution of the appropriate non-esterified fatty acid in methanol to the assay solution. This was done for control solutions that contained no bivalent cations and for solutions containing bivalent cations, both alone and in the presence of Ca^{2+} (Figure 1a). Conductimetric titration of solutions containing non-esterified fatty acid by sequential additions of zinc acetate established that Zn^{2+} did not form complexes with the octanoate ion until both concentrations exceeded 0.4 mM (results not shown).

Calcium buffer solutions were prepared by adding 1 mM nitrilotriacetic acid (NTA) to the standard assay buffer solution. Free Ca^{2+} concentrations were determined by conductimetric titration of sequential addition of 4 μl portions of 100 mM $CaCl_2$ to buffer solutions with and without 1 mM NTA. A difference curve (Figure 1b) was constructed and used to determine the value of the dissociation constant ($K_d = [Ca^{2+}][NTA]/[Ca^{2+}\cdot NTA]$), found to be 8 ± 1 μM, which was substantially larger than the value extrapolated from the published data [21].

Because all Ca^{2+} chelators have higher affinity for Zn^{2+} than for Ca^{2+}, free Ca^{2+} levels cannot be controlled in the presence of Zn^{2+} and, where necessary, were determined by conductimetric titration using EGTA and by comparing the level of PLA_2 activity with that determined for Ca^{2+}/NTA solutions. All solutions used in this work had free Ca^{2+} concentrations of 2 ± 0.5 μM.

RESULTS

Electrophoretic analysis showed that, in contrast with other cobra venoms, the venom of *Naja naja atra* contained only one major isoform of PLA_2 (Figure 2).

The aim of this study was to develop kinetic techniques to obtain complete data for Ca^{2+} activation of PLA_2 enzymes. From the reaction equation for the accepted mechanism whereby Ca^{2+} binding precedes substrate binding:

$$1/V = 1/V_{max.}\{1 + K_{Ca}/[Ca^{2+}](1 + [M]/K_m) + K_s/[S](1 + K'_{Ca}/[Ca^{2+}])(1 + [M]/K_m)\} \quad (1)$$

where K_{Ca} and K'_{Ca} are composite constants that determine the effect of Ca^{2+} on the substrate-independent and substrate-dependent terms: K_s is the conventional Michaelis constant and K_m is the dissociation constant for binding of an inhibitory metal ion M to the single Ca^{2+}-binding site.

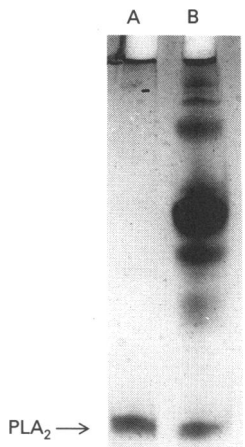

Figure 2 Gel electrophoresis of PLA_2 from *Naja naja atra* venom

PLA_2 from *Naja naja atra* venom was purified as described in the text, freeze-dried and analysed by electrophoresis on an alkali/urea gel containing 15% acrylamide, 0.8% bisacrylamide, 6 M urea with 2% ethanolamine as both gel and tank electrolyte. Migration was towards the anode, and the gel was stained with 0.1% Coomassie Blue for 15 min and destained with acetic acid/methanol/water (5:7:100, by vol.). Lane A represents 10 μl of the crude venom, and lane B 10 μl of the purified PLA_2.

This equation shows that the actions of all metal ion inhibitors should differ quantitatively, but not qualitatively, and be formally equivalent to decreasing the Ca^{2+} activation.

Inhibition by Ba^{2+} and Zn^{2+}

Studies of the action of the two bivalent cations, Ba^{2+} and Zn^{2+}, which were known to be the most effective inhibitors of most PLA_2 enzymes, gave completely unexpected results (Figure 3). The lowest Ca^{2+} concentration that could be used in the presence of Zn^{2+} was the residual level present in our buffers, namely 2 μM, and above a concentration of 1 mM no further increase in catalytic activity was observed. Between these two levels the enzymic activity increased by 7-fold, inhibition by Ba^{2+} fell 24-fold, but, in contrast, inhibition by Zn^{2+} increased by 4-fold. The difference in the effect of these two inhibitory ions was most clearly seen in the shape of the reaction progress curves, and the most striking feature is the accentuated curvature of those obtained with Zn^{2+} at high Ca^{2+} concentration. However, it should be noted that the curve eventually reaches the same end point as the Zn^{2+}-free responses, confirming the assay linearity. Intermediate results (not shown) were found for 20 μM and 200 μM Ca^{2+}. These results are consistent with Ba^{2+} acting as a classical competitive antagonist of Ca^{2+}, but Zn^{2+} clearly acts in a different fashion.

The enhanced curvature of the reaction time courses suggested that Zn^{2+} might be a slow-acting irreversible inhibitor of the enzyme, but preincubation of the enzyme with a high concentration of Zn^{2+} to achieve the same concentration (20 μM) after dilution into the assay solution did not affect the degree of inhibition or the shape of the progress curves.

The concentration-dependence of inhibition by Ba^{2+} showed the reciprocal relationship expected for competition with Ca^{2+} at a single essential site (Figure 4). In contrast, inhibition by Zn^{2+} was extremely weak at low Ca^{2+} concentration, but markedly biphasic at high Ca^{2+} concentration (Figure 5), indicating that Zn^{2+} competed very strongly when Ca^{2+} was present at high concentration, but decreased activity without abolishing it. The simplest interpretation of these data is that Zn^{2+} binds to the enzyme at both low and high Ca^{2+} concentration, but only inhibits strongly when the Ca^{2+} concentration was high, suggesting that Zn^{2+} displaces Ca^{2+} from a low-affinity, non-essential, activity-modulating site.

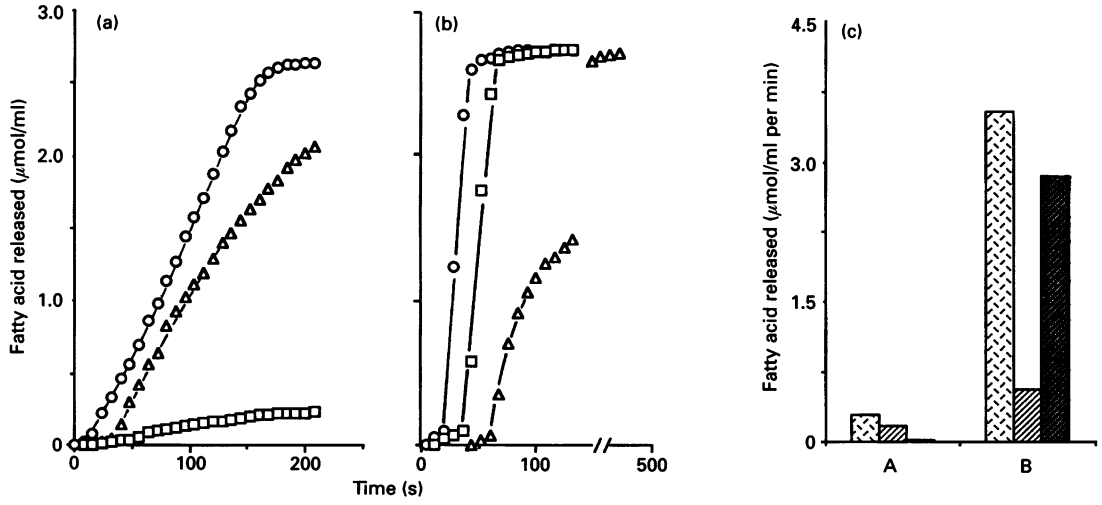

Figure 3 Effects of Ba^{2+} and Zn^{2+} on the time course of hydrolysis of DOPC by PLA_2 from *Naja naja atra*

The reactions were carried by adding 2 μg (approx. 80 nM) of PLA_2 to conductivity cells containing 2 ml of 10 mM triethanolamine/HCl buffer, pH 8.0, and 0.3 mM of DOPC with: (**a**) control with no added bivalent cations (○), or with 25 μM Zn^{2+} (△) or 0.5 mM Ba^{2+} (□); (**b**) 1 mM Ca^{2+} (○), 1 mM Ca^{2+} and 25 μM Zn^{2+} (△), or 1 mM Ca^{2+} and 0.5 mM Ba^{2+} (□). The histograms in (**c**) represent the initial rates of the hydrolysis reactions described above, with symbols as follows. Block A: (⊡), control (no added cations); (▨), with 25 μM Zn^{2+}; (■), with 0.5 mM Ba^{2+}. Block B: (⊡), with 1 mM Ca^{2+}; (▨), with 1 mM Ca^{2+} and 25 μM Zn^{2+}; (■), with 1 mM Ca^{2+} + 0.5 mM Ba^{2+}.

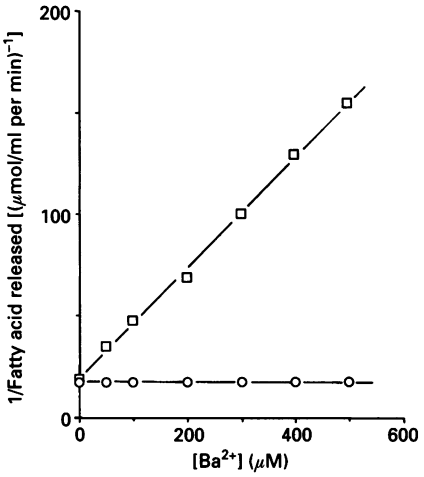

Figure 4 Inhibition of PLA_2 from *Naja naja atra* by Ba^{2+}

Plot of the reciprocal of initial rates of hydrolysis reactions carried out as described above as a function of different Ba^{2+} concentrations, (□) in the absence and (○) in the presence of 1 mM Ca^{2+}.

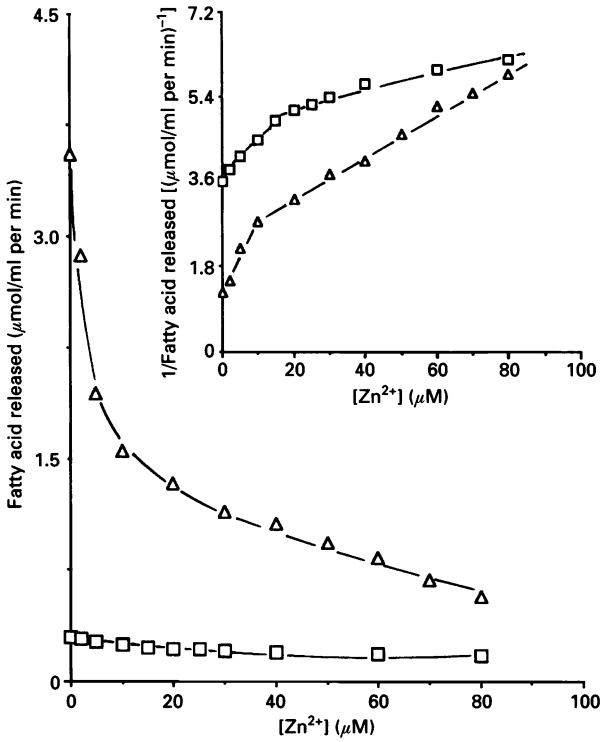

Figure 5 Inhibition of PLA_2 from *Naja naja atra* by Zn^{2+}

Initial rates of the hydrolysis of 0.3 mM DOPC were plotted as a function of Zn^{2+} concentration, (□) in the absence and (△) in the presence of 1 mM Ca^{2+}. The inset represents the reciprocal of initial rates of hydrolysis as a function of [Zn^{2+}]. The high-[Ca^{2+}] values (△) were multiplied by 4 for better comparison and clarity.

Comparison of the inhibitory potency of a variety of other bivalent cations gave a very clear division into two families, the competitive inhibitors, in which the order of potency was Ba^{2+} > $Pb^{2+} \gg Cu^{2+}$, and the non-competitive inhibitors, of which the only two examples were $Zn^{2+} > Cd^{2+}$ (results not shown). Ba^{2+} and Pb^{2+} are large ions [ionic radii 0.134 nm (1.34 Å) and 0.122 nm (1.22 Å) respectively], whereas Zn^{2+} and Cd^{2+} share co-ordination properties to a remarkable degree. Thus it would seem that size is the most important determinant of competitive behaviour, and specific co-ordination properties underly the non-competitive action.

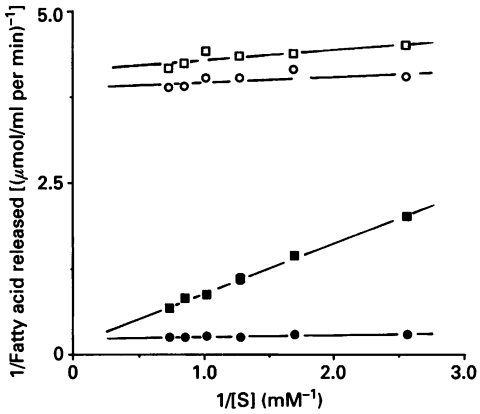

Figure 6 Effect of Zn^{2+} on the kinetic properties of PLA_2 from *Naja naja atra*

Reactions were carried out as described above, but with different concentrations of DOPC, and the data were analysed by a double-reciprocal plot ($1/v$ versus 1/[S]): ○, with 2 μM Ca^{2+}; □, with 2 μM Ca^{2+} and 25 μM Zn^{2+}; ●, with 1 mM Ca^{2+}; ■, with 1 mM Ca^{2+} and 25 μM Zn^{2+}.

Effect of substrate concentration on inhibition by Zn^{2+}

The dependence of activity on substrate concentration was studied at high and low Ca^{2+} concentration in the absence and presence of Zn^{2+}. The results (Figure 6) showed that activation by Ca^{2+} was almost entirely due to the elevation of the $V_{max.}$ term. The presence of Zn^{2+} at low Ca^{2+} concentration had very little effect on either the substrate-dependent or the substrate-independent terms. In marked contrast, the inhibitory action of Zn^{2+} at high Ca^{2+} concentration was highly dependent on substrate concentration. Because inhibition by Zn^{2+} was independent of substrate concentration at low Ca^{2+} concentration, this result suggests that Ca^{2+} and Zn^{2+} compete at a low-affinity modulating site and that the presence of bound substrate enhances the binding of Ca^{2+} to a greater degree than the binding of Zn^{2+}.

Concentration-dependence of Ca^{2+} activation

Most venom PLA_2 enzymes retain at least 10% of their activity in the DOPC assay in the absence of added Ca^{2+}, and this remains true even with the highest-quality laboratory distilled water and with extensively deionized substrates. The lowest Ca^{2+} level easily achieved in the absence of chelators is > 2 μM, and in order to obtain precise control over the required range a calcium buffer was required. Initial tests showed that this range was too high for EDTA/EGTA calcium buffers, but ideal for NTA, which was shown to have a K_d for Ca^{2+} of ~ 8 μM in our assay buffer. Thus by using 1 mM NTA it was possible to ignore endogenous Ca^{2+} without introducing gross errors. The null hypothesis, that the enzyme binds a single kinetically important

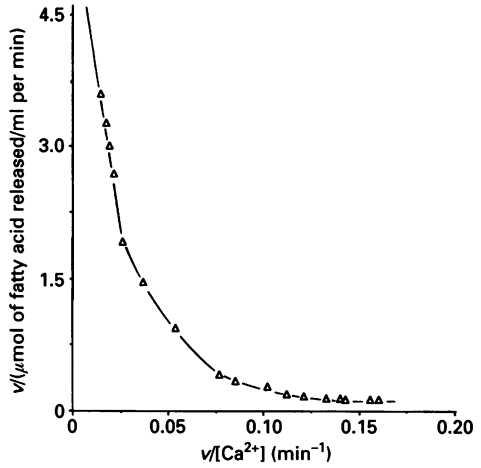

Figure 7 Ca^{2+}-dependence of PLA_2 from *Naja naja atra*

Standard hydrolysis reactions were carried out as in Figure 3, but in the presence of 1 mM NTA. Free Ca^{2+} concentrations were calculated from the data in Figure 1(b), and the results were analysed by plotting the initial rate v against $v/[Ca^{2+}]$, a construction analogous to the Eadie–Hofstee formulation.

Ca^{2+} ion, predicts linear plots using either the Lineweaver–Burk or the Eadie–Hofstee methods ($1/V$ versus $1/[Ca]$ or V versus $V/[Ca]$). The experimental data (Figure 7) consistently failed to confirm this prediction, and curve analysis is consistent with the presence of two distinct Ca^{2+}-binding sites with K_d values for Ca^{2+} of 2.7 μM and 125 μM, in which the essential Ca^{2+} ion supports 10% of the final maximum activity.

DISCUSSION

The results presented here centre around one major observation, that Zn^{2+}, known to be a powerful general inhibitor of PLA_2 enzymes, does not inhibit strongly at low Ca^{2+} concentration (Figures 3, 5 and 6), but does so at high Ca^{2+} concentration. This is strikingly opposed to the conventional behaviour when one ion antagonizes another. The observation is so dramatic and crucial that it calls into question the basic model for Ca^{2+} activation.

In marked contrast, the Ba^{2+} ion inhibits PLA_2 enzymes by a mechanism that has the full characteristics of competitive displacement of a single essential Ca^{2+} ion. It has been a matter of some interest to understand how the much smaller Zn^{2+} ion could bind with high affinity at the same site as the much larger Ba^{2+} ion, whereas intermediate-sized ions were less effective as inhibitors. The present results now show that the binding characteristics of these two ions are too dissimilar to be readily interpreted as antagonism at the same Ca^{2+}-binding site. Previous workers assumed that a single site was operative, and no previous kinetic analysis has been carried out in sufficient detail to provide a definitive test of mechanism. The data presented in this paper, although obtained with a different assay system, have nevertheless been obtained under reaction conditions that are typical of those employed by other workers.

Interpretation of the difference in inhibition kinetics of Ba^{2+} and Zn^{2+} leads to a choice between two possibilities: either that Zn^{2+} modifies the properties of the substrate, or else that Zn^{2+} binds to the enzyme at a site which is different from the essential Ca^{2+}-binding site. The evidence that this enzyme/substrate system has two kinetically distinct Ca^{2+}-binding sites comes from direct analysis of Ca^{2+} activation, where the data (Figure 7) are most easily interpreted by proposing that the enzyme has a high-affinity essential site and a lower-affinity modulating site which changes the maximum activity by a factor of nearly 10-fold.

Although the existence of a second Ca^{2+}-binding site is clear, its nature is not. There is strong circumstantial pressure to conclude that the second Ca^{2+}-binding site observed by crystallography is indeed the low-affinity modulating site. Nevertheless it is essential to bear in mind the complexity of interfacial phenomena seen in these systems and to consider the possibility that the second Ca^{2+}-binding site is associated with the bulk lipid substrate and that the kinetic effects of Zn^{2+} are actually produced at this site. We argue that saturable binding at this site could not be determined by bulk lipid, but could be produced by a trace level of an anionic impurity. Saturating binding of a bivalent metal ion to such an impurity could be detected with very high sensitivity by conductimetric analysis, but no detectable departure from linearity was ever observed when the substrate solution was titrated with either Ca^{2+} or Zn^{2+}. It should be noted that the purification of the substrate made extensive use of ion-exchange techniques and that the conductance of a 0.1 M solution of DOPC in methanol was indistinguishable from that of pure methanol.

There is little information to support the model that metal-ion binding by phospholipids could produce the observed competition phenomena. An early study of bivalent-cation binding to purified lecithin, using the sensitive method of charge reversal to measure the ability of different cations to neutralize a residual negative charge, produced the series of cation affinities: $Pb^{2+} > Cd^{2+} > Cu^{2+} > Zn^{2+} > Ca^{2+} > Mg^{2+} \gg Ba^{2+}$ [22]. The concentrations required for charge neutralization varied from 6 mM (Pb^{2+}) to 130 mM (Ba^{2+}), and are two orders of magnitude too high to be of possible significance here.

Thus the model of a modulating metal-ion binding site formed by the substrate where Zn^{2+} alone of bivalent cations is a specific antagonist for Ca^{2+} has no supporting evidence. Although it cannot be ruled out that Zn^{2+} acts at the lipid interface, it is extremely difficult to propose a satisfactory model to explain the Ca^{2+}-dependence. Furthermore, there is very clear evidence that Zn^{2+} interacts directly with amino acid residues in PLA_2 enzymes [9,10].

If the site of action of Zn^{2+} is at the enzyme, then it is possible to place two severe restraints on the mechanism of inhibition, the first being that it cannot replace Ca^{2+} at the essential site and the second that it must displace Ca^{2+} from a site which modulates, but does not determine, the catalytic activity.

This model can be summarized by proposing that the enzyme has three active forms, PLA_2Ca^{2+} (I), $PLA_2Ca^{2+}\cdot Zn^{2+}$ (II), and $PLA_2(Ca^{2+})_2$ (III), where (I) and (II) have very similar kinetic properties and give rise to approx. 10% of the catalytic activity of (III). However there is an anomalous feature, which is clear from Figure 6. The substrate affinity of forms (I) and (III) is high, but that of (II) is indeterminate, appearing to be low when Ca^{2+} concentration is high and high when Ca^{2+} concentration is low. This can be rationalized by assuming that the basic catalytic properties of (I) and (II) are very similar and that Zn^{2+} inhibits by displacing the second Ca^{2+} ion from (III). If the binding of ions at the second site is influenced by the substrate concentration, and if Zn^{2+} is less sensitive to this component than Ca^{2+}, then Zn^{2+} should displace Ca^{2+} in a substrate-dependent manner.

The present results carry the implication that all other PLA_2 enzymes known to be inhibited by Zn^{2+} and Ba^{2+} will also possess two separate ion-binding sites. But, because there is no evidence that the majority of PLA_2 enzymes have two Ca^{2+}-binding sites, any such general conclusion must be viewed with caution.

Nevertheless, it may well apply to all PLA_2 enzymes for which Zn^{2+} inhibition increases with increased Ca^{2+} concentration.

In summary, inhibition of PLA_2 enzymes by Zn^{2+} is, in contrast with inhibition by Ba^{2+}, a phenomenon of physiological interest, because Zn^{2+} is present in effective concentrations in body fluids and in venoms. This paper shows that inhibition by Zn^{2+} is qualitatively unlike inhibition by Ba^{2+}, and the major effects cannot be attributed to the displacement of Ca^{2+} from a single essential site.

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REFERENCES

1 Dijkstra, B. W., Drenth, J. and Kalk, K. H. (1981) Nature (London) **289**, 604–606
2 Dijkstra, B. W., Kalk, K. H., Hol, W. G. J. and Drenth, J. (1981) J. Mol. Biol. **147**, 97–123
3 Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gleb, M. H. and Sigler, P. B. (1990) Science **250**, 1541–1546
4 White, S. P., Scott, D. L., Otwinoski, Z., Gleb, M. H. and Sigler, P. B. (1990) Science **250**, 1560–1563
5 Wells, M. A. (1972) Biochemistry **11**, 1030–1041
6 Wells, M. A. (1973) Biochemistry **12**, 1080–1085
7 Tsai, T. C., Hart, J., Jiang, R. T., Bruzik, K. and Tsai, M. D. (1985) Biochemistry **24**, 3180–3188
8 Anderson, T., Drakenberg, T., Forsen, N., Weiloch, T. and Lindstrom, M. (1981) FEBS Lett. **123**, 115–117
9 Wells, M. A. (1974) Biochemistry **13**, 2248–2257
10 Wells, M. A. (1974) Biochemistry **13**, 2265–2268
11 Donne-Op den kelder, G. M., de Haas, G. H. and Egmond, M. R. (1983) Biochemistry **22**, 2470–2478
12 van den Bergh, C. J., Bekkers, A. C. A. P. A., Verheij, H. M. and de Haas, G. H. (1989) Eur. J. Biochem. **182**, 307–313
13 Ahmad, T. and Lawrence, A. J. (1993) Toxicon **31**, 1279–1291
14 Chettibi, S. and Lawrence, A. J. (1989) Toxicon **27**, 781–787
15 Patel, K. M., Morrisett, J. D. and Sparrow, J. T. (1979) J. Lipid Res. **20**, 674–677
16 Lawrence, A. J. (1971) Eur. J. Biochem. **18**, 221–225
17 Lawrence, A. J. and Moores, G. R. (1972) Eur. J. Biochem. **24**, 538–546
18 Lawrence, A. J., Moores, G. R. and Steele, J. (1974) Eur. J. Biochem. **48**, 277–286
19 Drainas, D. and Drainas, C. (1985) Eur. J. Biochem. **151**, 591–593
20 Mezna, M. and Lawrence, A. J. (1994) Anal. Biochem. **218**, 370–376
21 Sillen, L. G. and Martell, A. E. (eds.) (1964) Stability Constants of Metal-Ion Complexes: Special Edition no. 17, Metcalfe and Cooper, London
22 Bungenberg de Jong, H. G. (1965) in Colloid Science, vol. 2 (Kruyt, H. R., ed.), p. 281, Elsevier Publishing Co., Amsterdam

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