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MACROPHAGE ARACHIDONATE-MOBILIZING PHOSPHOLIPASE A_2: ROLE OF Ca^{2+} FOR MEMBRANE BINDING BUT NOT FOR CATALYTIC ACTIVITY

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A recently purified Ca^{2+}-dependent intracellular phospholipase A_2 from spleen, kidney and macrophage cell lines is activated by Ca^{2+} at concentrations achieved intracellularly. Using enzyme from the murine cell line J774 we here demonstrate the formation of a ternary complex of phospholipase, $^{45}Ca^{2+}$ and phospholipid vesicle, and provide evidence for a single Ca^{2+}-binding site on the enzyme involved in its vesicle binding. Although Ca^{2+} binds to and functions as an activator of the enzyme, this ion does not appear to be involved in its catalytic mechanism, since enzyme brought to the phospholipid vesicle by molar concentrations of NaCl or NH_4^+ salts exhibited Ca^{2+}-independent catalytic activity.

The mobilization of arachidonate is thought to be due to activation of an intracellular phospholipase A_2 catalyzing the rate-limiting step in the biosynthesis of eicosanoids (1, 2). We earlier identified a high molecular mass phospholipase A_2 in murine macrophages (3) with a Ca^{2+}-dependence, an arachidonoyl-preference and an ability to be activated via protein kinase C, strongly suggesting that it is responsible for the regulated mobilization of arachidonate in macrophages. Recently, related enzymes with similar enzymatic properties have been purified from rat kidney (4), mouse spleen (5), the murine macrophage cell lines RAW264.7 (6) and J774 (5) and the human cell line U937 (7, 8). After cloning and sequencing of cDNA (9, 10) and partial sequencing at the protein level (5), it now appears that they represent species homologues of a single 85 kDa phospholipase .

Abbreviation: PLA_2-85, 85 kDa intracellular phospholipase A_2.

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In the well characterized extracellular phospholipases A_2, such as that from pancreas, Ca^{2+} is necessary for catalytic activity. The ion binds at a defined site located within the active site of the enzyme (11) and takes part in the catalytic mechanism (12). Using purified PLA_2-85 from J774 cells we here report on the role of Ca^{2+} for phospholipid interaction and catalytic activity of this intracellular enzyme.

MATERIALS AND METHODS

PLA_2-85 from the macrophage cell line J774 was purified as previously described (5). For experiments with radioactive Ca^{2+}, purified PLA_2-85 was freed of EDTA on a Sephadex G-25 column equilibrated in 80 mM KCl, 10 mM Hepes, pH 7.4 and 10 % glycerol [v/v] (buffer A).

The standard mixture for assay of PLA_2-85 contained 0.15 - 0.5 nmol of 1-stearoyl-2-[^{3}H]arachidonoyl-phosphatidylcholine (20 000 dpm) as sonicated vesicles, $CaCl_2$ (625 nmol; equal to 0.4 mM free Ca^{2+}), fatty acid depleted bovine serum albumin (100 μg) and buffer (80 mM KCl, 1 mM EDTA and 10 mM Hepes, pH 7.4) to a final volume of 525 μl. The mixture was incubated for 30 min at 37 °C . Lipids were extracted, followed by separation of arachidonic acid and phospholipid on silicic acid columns as previously described (3).

Radiolabeled phosphatidylinositol was from Du Pont-New England Nuclear while other radiolabeled phospholipids and $^{45}Ca^{2+}$ were from Amersham. NaCl (pro analysi) and NH_4Cl (suprapur) were from Merck, Germany, while all other ammonium salts (AnalaR) were from BDH, England.

RESULTS

Previous results indicated that macrophage PLA_2-85 interacts avidly with highly curved phosphatidylcholine vesicles (5). If this Ca^{2+}-dependent interaction were sufficiently stable to survive a chromatographic separation, possible binding of Ca^{2+} to PLA_2-85 should be demonstrable using even pmolar amounts of enzyme. PLA_2-85 was therefore incubated with radioactive Ca^{2+} (30 μM) and sonicated phosphatidylcholine vesicles followed by gel chromatography. Figure 1 A shows that bound Ca^{2+} was indeed recovered in an enzyme-vesicle complex, while no Ca^{2+} remained bound when either enzyme or vesicles were omitted (Fig. 1 B-C). The amount of $^{45}Ca^{2+}$ bound was directly proportional to the amount of enzyme in the incubation and the complex was surprisingly stable, since it was not fully dissociated by short-term exposure (5 min) to 2 mM EDTA prior to chromatography (not shown). Only minor amounts of $^{45}Ca^{2+}$ and PLA_2-85 activity eluted between the void volume and the position for free enzyme (Fig. 1 A), indicating that desorption from the vesicles during chromatography was limited. However, the fact that enzyme activity was detected in the void-volume complex indicates that catalytically active enzyme can desorb from the vesicles. An accurate determination of the amount of enzyme in these fractions, from measurment of enzyme activity, could not be made because of the presence of unlabeled preincubation vesicles. However, under the assumption that all of the applied PLA_2-85, except for that recovered

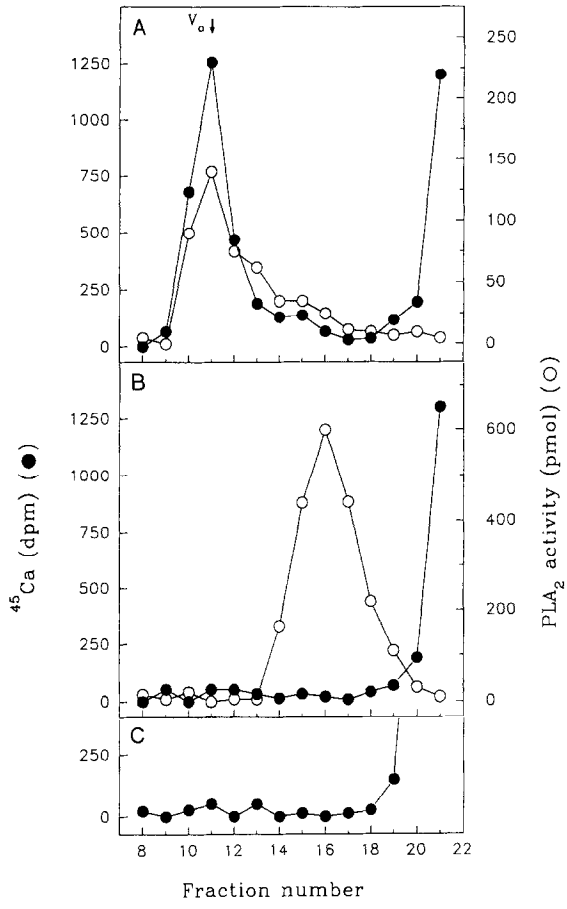

Figure 1

Binding of radioactive Ca^{2+} to PLA_2-85.

(A) 0.2 μg of PLA_2-85 (free of EDTA) was incubated with 8 μCi [$^{45}Ca^{2+}$] (specific act. 2.19 mCi/84 μg), 300 μg fatty acid depleted bovine serum albumin and 50 nmol of sonicated phosphatidylcholine vesicles in a final volume of 350 μl. After incubation for 5 min at 20 °C the mixture was at 4 °C loaded onto a Sephacryl S-300 column (bed volume 13 ml) previously equilibrated in buffer A without EDTA but supplemented with 150 mM NaCl and bovine serum albumin (10 μg/ml) . The column was eluted at a flow rate of 0.5 ml/min and 0.6 ml fractions were collected. [$^{45}Ca^{2+}$] in the fractions was determined by liquid scintillation spectrometry and PLA_2-85 activity was determined as described in Materials and methods. The void volume of the column was determined with sonicated vesicles of [^{3}H]arachidonoyl-phosphatidylcholine. (B) As in A, but without sonicated vesicles. (C) As in A but without PLA_2-85.

as free PLA_2-85, bound to the phosphatidylcholine vesicles, five separate experiments revealed binding of 0.7 ± 0.2 mol $^{45}Ca^{2+}$/ mol PLA_2-85. It is therefore likely that a single Ca^{2+}-binding site on the enzyme is involved in its interaction with phospholipid. The presence of additional sites, with rapidly reversible binding of Ca^{2+}, can of course not be excluded using the present experimental approach.

Also Mg^{2+} was found to cause hydrolysis of phosphatidylcholine with a very similar time course as that seen with Ca^{2+} (Fig. 2). The hydrolysis increased gradually up to 7 mM

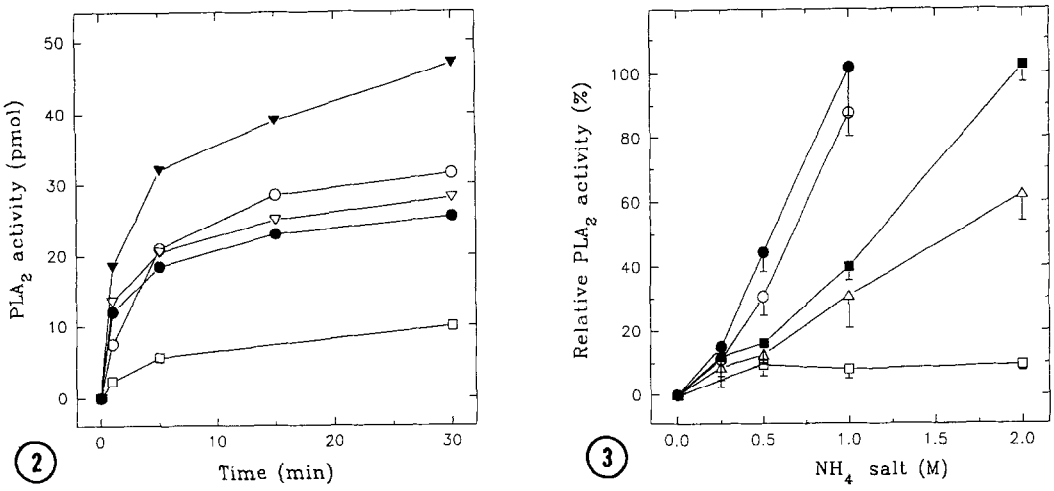

Figure 2

Time course of monovalent salt- and divalent cation -induced PLA_2-85 activity.

Purified PLA_2-85 was assayed with 200 pmol of [^{3}H]arachidonoyl-phosphatidylcholine in the presence of 0.4 mM free Ca^{2+} ( ); 2 M NaCl and 5 mM EDTA ( ); 0.9 M $(NH_4)_2SO_4$ and 5 mM EDTA ( ); 0.9 M $(NH_4)_2SO_4$ and 0.4 mM free Ca^{2+} ( ); 7 mM Mg^{2+} and 1 mM EGTA ( ).

Figure 3

Activation of PLA_2-85 by different ammonium salts.

Purified PLA_2-85 was assayed with 200 pmol [^{3}H]arachidonoyl-phosphatidylcholine for 30 min with, $(NH_4)_2SO_4$ ( ); $(NH_4)H_2PO_4$ ( ); NH_4CH_3COOH ( ); NH_4COOH ( ); NH_4Cl ( ). All incubations were performed in the presence of 5 mM EDTA. Results are mean values ± S.D. ($n \geq 3$) and are expressed as relative PLA_2-85 activity with 100 % equal to the hydrolysis seen in the presence of 0.4 mM free Ca^{2+}.

Mg^{2+} with no further increase at 0.3 M Mg^{2+} and was about 40 % of that obtained with Ca^{2+}. This could indicate low affinity binding of Mg^{2+} to the Ca^{2+}-binding site demonstrated above. The physiological importance of such an interaction is not clear but cytosolic Mg^{2+} might contribute to the maintenance of a basal activity of the enzyme as we found a low but significant activity in the presence of 1.9 mM Mg^{2+} (approx. 5 % of that with Ca^{2+}). Activation by Mg^{2+} has also been reported for PLA_2-85 from the monocytic cell line THP1 (13).

We previously showed that arachidonate-labeled phospholipids in macrophage membranes were hydrolyzed at a lower rate and with a more linear time course than sonicated phospholipid vesicles or sonicated macrophage membranes (5). This suggested that the Ca^{2+}-dependent interaction between PLA_2-85 and its physiological substrate is less stable than that with highly curved sonicated vesicles. In agreement with this suggestion, no stable complex with macrophage membranes could be demonstrated by gel chromatography after incubation in either 30 μM or 1 mM Ca^{2+}; all of the PLA_2-85 applied eluted as free enzyme (not shown).

In order to assess whether Ca^{2+} or other divalent cations play an indispensable role in the catalytic mechanism of PLA_2-85, attempts were made to provoke interaction of the

Table I

Hydrolysis of phosphatidylinositol in response to Ca^{2+} and ammonium salts

	PLA_2-85 activity (% hydrolysis)
$CaCl_2$	0.5 ± 0.2
$(NH_4)_2SO_4$	6.2 ± 0.7
$(NH_4)_2SO_4 + CaCl_2$	21.6 ± 0.1
NH_4Cl	0.6 ± 0.1
$NH_4Cl + CaCl_2$	5.0 ± 0.3

PLA_2-85 was assayed with 150 pmol of [^{14}C]arachidonoyl-phosphatidylinositol for 30 min in the presence of $CaCl_2$ (0.4 mM free Ca^{2+}), $(NH_4)_2SO_4$ (1.0 M), NH_4Cl (2.9 M) or combinations of these salts as indicated. Data represent mean ± S.D. from three separate experiments. For comparison, the same amount of PLA_2-85 resulted in 18 % hydrolysis when assayed in the presence of 0.4 mM free Ca^{2+} with 150 pmol [^{3}H]arachidonoyl-phosphatidylcholine as substrate.

enzyme with phospholipid vesicles independently of divalent cations. NaCl and $(NH_4)_2SO_4$ at molar concentrations in the presence of 5 mM EDTA were found to induce hydrolysis of phosphatidylcholine with a similar rate as Ca^{2+} (Fig. 2). In the presence of both Ca^{2+} and $(NH_4)_2SO_4$ the hydrolysis was further increased compared to either alone (Fig. 2). Different ammonium-salts were then compared for their ability to induce PLA_2-85 activity. $(NH_4)H_2PO_4$ was almost as potent as $(NH_4)_2SO_4$ while ammonium formate and ammonium acetate were somewhat less potent and NH_4Cl was a poor inducer of PLA_2-85 activity (Fig. 3).

Pure phosphatidylinositol vesicles were earlier shown to be hydrolyzed very slowly in the presence of Ca^{2+} by PLA_2-85 and further experiments indicated that this was due to poor binding of the enzyme to negatively charged vesicles (5). As shown in Table I, $(NH_4)_2SO_4$ induced more extensive hydrolysis of pure phosphatidylinositol vesicles than Ca^{2+} and a more than 3-fold further increase in hydrolysis was seen in the presence of both $(NH_4)_2SO_4$ and Ca^{2+}. A pronounced increase in Ca^{2+}-dependent hydrolysis was seen also with NH_4Cl, although NH_4Cl caused little hydrolysis in the absence of Ca^{2+}.

DISCUSSION

Ca^{2+} is a potent activator of PLA_2-85 and previous studies have indicated that Ca^{2+} causes translocation of the enzyme to cellular membranes (14, 15). However, binding of Ca^{2+} to the enzyme has not been demonstrated and the possibility that membrane translocation reflects substrate interaction at the active site, with a potential role for Ca^{2+}

in the catalytic mechanism, has not been excluded. The present study demonstrates binding of Ca^{2+} to PLA_2-85 from J774 cells and provides evidence for a single Ca^{2+}-binding site intimately involved in the interaction of the enzyme with a phospholipid bilayer. Clark et al. (9) recently showed that a 140 amino acid fragment from the N-terminal part of PLA_2-85 from U937 cells translocates to membranes in response to Ca^{2+}. The authors suggested that a sequence motif common to PLA_2-85 and certain other proteins (e.g. protein kinase Cγ and phospholipase Cγ1), some of which are known to translocate to membranes in response to Ca^{2+}, is responsible for the binding. However, an apparent discrepancy is that PLA_2-85 binds well to neutral, but less well to anionic phospholipid vesicles, while protein kinase C and several other proteins exhibiting Ca^{2+}-dependent phospholipid binding require anionic phospholipids.

Ammonium salts were found to induce PLA_2-85 activity in the absence of Ca^{2+} with $(NH_4)_2SO_4$ being the most potent and NH_4Cl the least potent (less than 10 % of that seen with $(NH_4)_2SO_4$). The order in which these salts induce PLA_2-85 activity is in agreement with their ability to strengthen hydrophobic interactions. If NaCl acted merely by the same mechanism, one would not expect this salt to be more than 5 times as potent as NH_4Cl. One may therefore speculate that Na^+, at a 10^6 times higher concentration than Ca^{2+} can interact with the Ca^{2+}-binding site and thereby promote membrane interaction of PLA_2-85. Interaction with the Ca^{2+}-binding site is also a likely explanation for the activation by millimolar concentrations of Mg^{2+}.

In the case of anionic phosphatidylinositol vesicles, $(NH_4)_2SO_4$ induced significantly higher activity than Ca^{2+} and also promoted Ca^{2+}-induced activation to a greater extent than seen with phosphatidylcholine. Ammonium salts, as well as NaCl, may here in addition act by reducing the negative surface potential and thereby facilitate the binding of enzyme, both in the presence and absence of Ca^{2+}.

Salt-induced activation has recently been demonstrated for an intracellular 30 kDa phospholipase A_2 from sheep platelets (16). The authors also reported that phospholipase A_2 from snake (Naja naja naja) and bee venom did not express catalytic activity in the presence of NaCl. Since pancreatic phospholipase A_2 has been shown to respond to 3 M NaCl, in the presence of Ca^{2+}, with an 80-fold increase in activity against phosphatidylcholine with 6-8 carbon chains (17), we tested the effect of salts on pancreatic phospholipase A_2 acting on sonicated vesicles of oleoyl-phosphatidylcholine. No enzyme activity was observed in response to NaCl or ammonium salts, nor was there any increase in Ca^{2+}-induced activity by molar concentrations of these salts.

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