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Responses of purified phospholipases A_2 to phospholipase A_2 activating protein (PLAP) and melittin

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The role of the phospholipase A_2 (PLA_2) stimulating protein PLAP in the regulation of PLA_2 activity was assessed by determination of the effects of PLAP on two purified PLA_2s. An approx. 14 kDa enzyme was purified from mouse thymoma cells, EL-4 cells, by cation ion exchange HPLC and immunoaffinity HPLC (with antiserum to the N-terminal sequence of an inflammatory exudate PLA_2). An approx. 110 kDa enzyme was purified from mouse mammary carcinoma derived cells by sequential hydrophobic, anion exchange, hydroxyapatite and gel filtration HPLC. Neither PLAP nor melittin, an immunologically related PLA_2 stimulating peptide from bee venom, increased the activity of the high molecular weight enzyme. In contrast, there was more than a 20-fold stimulation of the low molecular weight PLA_2 by PLAP and an approx. 5-fold stimulation by melittin. The stimulation of enzyme activity by PLAP was observed at a protein to phospholipid ratio of $1:10^6$ while the ratio of melittin to phospholipid was 1:3. Thus, PLAP mediated stimulation of PLA_2 activity may include an interaction between PLAP and the enzyme, in contrast to melittin stimulation, which involves interactions between melittin and phospholipid.

Introduction

The availability of free arachidonic acid is an important component in the regulation of eicosanoid biosynthesis [1,2]. Phospholipase A_2 (PLA_2) has a central role in the generation of free arachidonic acid for eicosanoid biosynthesis by hydrolysis of phospholipids with an arachidonoyl group in the *sn*-2 position [2,3]. In view of the importance of PLA_2 in the regulation of eicosanoid synthesis, as well as the participation of PLA_2s in diverse cellular functions [3–8], it is likely that specific PLA_2s have distinctive functions. PLA_2s with varying properties have been characterized from different tissues and cell types. Small molecular weight PLA_2s, approx. 12–15 kDa, have been isolated from lung, gastric mucosa and spleen; these PLA_2s are classified as group I PLA_2s on the basis of immunological cross-reactivity [9–11]. Pancreatic PLA_2 is a group I PLA_2. Other PLA_2s have been identified which are similar in size but differ in structure and biochemical characteristics [12–15]. Some are classified as members of the group II PLA_2s on the basis of their immunological cross-reactivity and/or amino acid sequences [16]. Group II enzymes include human synovial fluid and platelet PLA_2s, which have been shown to be identical on the basis of their cDNAs [12,13]. These enzymes have high p*I* values and require Ca^{2+} for activity, typically in the millimolar range [12–16]. Recently, a high molecular weight PLA_2 has been characterized which is distinctive from the low molecular weight PLA_2s on the basis of its predicated amino acid sequence, requirement for less than micromolar Ca^{2+} for activity and translocation between the soluble and membrane fractions as a function of Ca^{2+} concentration [17–19]. In addition, other PLA_2s have been described whose activity is Ca^{2+} independent and still others are inhibited by Ca^{2+} [20,21]. Thus, there are multiple PLA_2s with varying structural and functional characteristics.

The multiplicity of PLA_2s may provide for a diversity in function and for specificity in the regulation of enzyme activity in response to a wide range of extracellular signals. Regulation of PLA_2 activity has been observed in response to treatment of cells with a variety of ligands including interleukin-1, tumor necrosis

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Abbreviations: PLA_2, phospholipase A_2; PLAP, phospholipase A_2 activating protein; HPLC, high-performance liquid chromatography; BPI, bactericidal permeability increasing protein.

factor, bradykinin, norepinephrine and epidermal growth factor [22–33]. Agonist mediated stimulation of PLA_2 activity appears to occur via a number of different mechanisms. These include increased activity in response to increased intracellular Ca^{2+} concentration [5,19], long term stimulation of PLA_2 activity due to increased PLA_2 mRNA and protein [23], and rapidly increased PLA_2 activity due to increased levels of mRNA and protein for the PLA_2 activating protein, PLAP [24,33–37]. Specificity of regulation of PLA_2 is illustrated by the finding that the bradykinin stimulation of arachidonic acid release in endothelial cells is via a PLAP independent mechanism whereas leukotriene D_4 stimulation of this release in the same cells is PLAP dependent [36]. PLAP was originally identified on the basis of its immunological cross-reactivity with antibodies to the bee venom PLA_2 stimulating peptide, melittin [34]. Melittin stimulates some, but not all, PLA_2s [38]. In this study, Ca^{2+} dependent high and low molecular weight PLA_2s, which are likely candidates for participation in regulation of free arachidonic acid levels, were purified from mouse cells and their potential regulation by PLAP was examined by determination of their responsiveness to PLAP and melittin.

Materials and Methods

Cells and cell culture

EL-4 murine T cells (EL-4.IL-2) were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT). MTV-L/BALB Cl 2 cells, a mouse mammary carcinoma derived cell line with high PLA_2 activity [39], were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 3 μg/ml insulin (Sigma, St. Louis, MO). Tumors were induced by subcutaneous injection of BALB/c female mice with 10^6 MTV-L/BALB Cl 2 cells.

Isolation and purification of a low molecular weight PLA_2 from EL-4 cells

EL-4 cells were harvested by centrifugation ($100 \times g$, 20 min), resuspended in 10 volumes of 50 mM Hepes (pH 8.0), plus a cocktail of protease inhibitors (1 mM benzamidine, 100 μg/ml bacitracin, 5 μg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. The sonicate was centrifuged, $100\,000 \times g$ for 30 min, and the resultant supernatant fraction was applied to a Mono S cation exchange column (10 mm × 10 cm) (Pharmacia LKB Biotechnology, Piscataway, NJ) at a flow rate of 1 ml/min. The column was eluted with a linear gradient of 0 to 1.0 M NaCl in 50 mM Hepes (pH 8.0). Fractions were collected and assayed for PLA_2 activity as previously described [40]. Briefly, the reactions were initiated by the addition of substrate (1-stearoyl-2[^{3}H] arachidonoyl-*sn*-glycero-3-phosphocholine, 52 mCi/mmol) (New England Nuclear, Boston, MA) which had been first evaporated to dryness under nitrogen, then dissolved in deoxycholate (5 mM) using a bath sonicator. The reaction volume was a total of 50 μl and consisted of: Tris, 200 mM (pH 9.0), NaCl, 100 mM, $CaCl_2$, 1 mM, and 10 μl of each column fraction. The final concentration of substrate in the reaction was 10 μM and the final concentration of deoxycholate in the reaction was 1 mM. The reactions were incubated at 37°C for 60 min, then terminated by Bligh and Dyer extraction. Fractions 22 to 26 were pooled and further purified on an HPLC immunoaffinity column prepared by coupling antibodies made to the N-terminal sequence (first 18 amino acids, synthesized by *t*-butoxycarbonyl chemistry) of an inflammatory exudate PLA_2 [41] to an HPLC column (Beckman EP, Palo Alto, CA). The pooled fractions were applied to the immunoaffinity column and the column was extensively washed with 50 mM Hepes (pH 8.0), 0.05% Tween 20 (v/v) until the absorbance at 280 nm returned to baseline; a flow rate of 0.5 ml/min was used for all steps. The bound material was eluted with 50 mM sodium acetate (pH 3.1). Fractions were collected at 4 min intervals, adjusted to pH 7.0 with 2.0 M Tris, assayed for PLA_2 activity, and stored at 4°C. Alternatively, the enzyme was concentrated 10 fold by ultrafiltration and mixed with glycerol (50%, v/v) and stored at −20°C for up to 1 month without loss of appreciable activity.

Isolation and purification of a high molecular weight PLA_2 from mouse mammary carcinomas

The high molecular weight PLA_2 was isolated from tumors excised from animals injected 2 weeks previously with MTV-L/BALB Cl 2 cells. Tumors were homogenized in 5 volumes of homogenization buffer (30 mM Tris (pH 7.8), 2 mM EDTA, 120 mM NaCl plus the cocktail of proteinase inhibitors used above) with a Polytron, then 5 volumes of 2.0 M $(NH_4)_2SO_4$ in homogenization buffer were added to the homogenate. The resulting homogenate was centrifuged, $10\,000 \times g$ for 20 min and the resulting supernatant fraction was centrifuged at $100\,000 \times g$ for 30 min. The high speed supernatant fraction was applied to an Ether-5PW HPLC column (50 mm × 20 cm) (TosoHaas, Philadelphia, PA). The column was eluted with a linear gradient, initial buffer 1.0 M $(NH_4)_2SO_4$ in 0.1 M Tris (pH 8.2), and final buffer 0.1 M Tris (pH 9.5) at a flow rate of 15 ml/min. Fractions were collected at 1 min intervals and assayed for activity as previously described using 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine (52 mCi/mmol, New England Nuclear) as the substrate [39]. Briefly, the

reaction volume was 50 μl and included: Tris, 220 mM (pH 9.5), NaCl, 100 mM, dithiothreitol, 1 mM, 0.26 mg/ml glycerol, 20% (v/v) and 8.15 μM substrate, which was added last to initiate the reactions. The reactions were incubated at 34°C for 2 min then terminated by Blygh and Dyer extraction. Fractions 40 to 50 were pooled, dialyzed overnight against 20 mM Tris (pH 8.0), 2 mM EDTA, 5 mM dithiothreitol, buffer A, and then chromatographed on an anion exchange column (20 mm × 20 cm DEAE-3SW column, TosoHaas). The sample was loaded on the column in 0.15 M NaCl in buffer A and the column was eluted with a gradient of 0.15 to 0.75 M NaCl in buffer A at a flow rate of 15 ml/min. Fractions 50 to 55, the single peak of PLA_2 activity, were pooled and concentrated by ultrafiltration (YM 30 membrane, Amicon, Beverly, MA). The concentrate was diluted with 10 volumes of 10 mM $KHPO_4$ (pH 6.8), reconcentrated and then, immediately before chromatography, rediluted with 10 mM $KHPO_4$ (pH 6.8) and $CaCl_2$, final concentration 0.01 mM $CaCl_2$. The sample was applied to an HCA column (15 mm × 15 cm) (Rainin, Woburn, MA) and eluted with a linear gradient of 0.01 to 0.50 M $KHPO_4$ (pH 6.8). A flow rate of 5 ml/min was used and fractions were collected at 1 min intervals in tubes containing EDTA and EGTA such that the final concentration of each was 1 mM. Fractions 25 to 35 were pooled and concentrated by ultrafiltration (YM 30 membrane), resuspended in the buffer used for gel filtration, described below, and reconcentrated by ultrafiltration. The concentrated sample was chromatographed on two gel filtration columns (15 mm × 25 cm), coupled in tandem (Zorbax Bio Series GF-450 and GF-250, MAC-MOD Analytical, Chadds Ford, PA) in 0.5 M NaCl, 20 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol. The peak of PLA_2 activity, fractions 20 to 24, was concentrated by ultrafiltration, diluted with an equal volume of glycerol and stored at −20°C.

Protein analyses

Protein was measured by the method of Bradford [42] using reagents obtained from Bio-Rad (Richmond, CA) with bovine serum albumin (Sigma) as standard. The low molecular weight PLA_2 was radiolabeled by iodination using ^{125}I from New England Nuclear (Boston, MA) and with chloramine T (IODO-BEADS, Pierce, Rockford, IL) as described by the manufacturers. The radioactive protein was desalted by gel filtration with Sephadex G10 (Pharmacia LKB Biotechnology) and analyzed by polyacrylamide gel electrophoresis (15% gel)/autoradiography. The high molecular weight PLA_2 was analyzed by polyacrylamide gel electrophoresis, 7.5% gel, with a PhastSystem (Pharmacia). Protein was detected by silver staining.

PLAP purification and assays for phospholipase stimulatory activity

PLAP was purified as previously described from $BC3H_1$ cells using a combination of affinity and gel filtration chromatography [34]. Purity of the PLAP was assesed by SDS-PAGE, and determined to be free from contaminating proteins. Stimulatory assays were performed as previously described [34]. Briefly, the reactions were performed as described for each of the phospholipase A_2 enzymes above except that PLAP, or melittin, was added to the reaction immediately prior to the addition of substrate.

Results

Purification of low molecular weight PLA_2

The low molecular weight PLA_2 was purified from the high speed supernatant fraction from EL-4 cells. The sample was first separated by cation exchange chromatography, which resolved the PLA_2 activity into two major fractions, one which either did not bind or only weakly bound to the column and the second fraction which was eluted from the column with high salt (Fig. 1A). In order to isolate a PLA_2 which was immunologically related to the low molecular weight group II PLA_2s, the following strategy was imple-

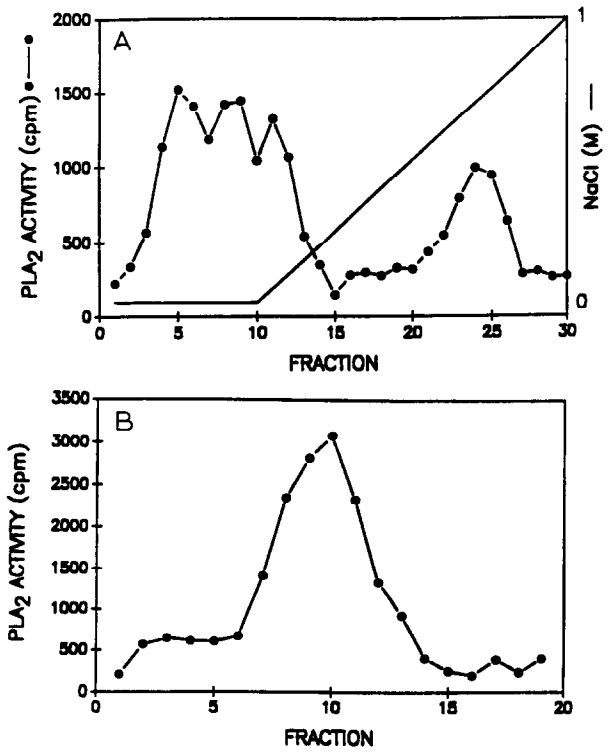

Fig. 1. Purification of a low molecular weight PLA_2. (A) Cation exchange chromatography of the high speed supernatant fraction from EL-4 cells; PLA_2 activity, cpm (•); sodium chloride, M (------); (B) Immunoaffinity HPLC of the PLA_2 from cation exchange chromatography, symbols as in A.

mented: (i) to screen the cation exchange chromatography fractions for cross-reactivity with antibodies which would recognize group II PLA_2s, (ii) to prepare an immunoaffinity HPLC column with these antibodies, and (iii) to chromatograph cross-reactive fractions from the Mono S column on the immunoaffinity column. Antisera to a peptide homologous to the first 18 amino acids of the N-terminal sequence of an inflammatory exudate PLA_2 [41] were generated and tested for cross-reactivity, as this sequence appears to be conserved in a number of PLA_2s reported to date [16]. Only the high salt eluting PLA_2 fraction from the Mono S chromatography step exhibited cross-reactivity (data not shown). This fraction was subjected to immunoaffinity HPLC. A single peak of PLA_2 activity was observed to elute from the immunoaffinity HPLC column (Fig. 1B). No PLA_2 activity was found in the flow through fractions. No ultraviolet absorbance at 280 nm was observed in the fractions with PLA_2 activity. Since there was insufficient material to accurately measure the protein content of the purified PLA_2, the specific activity of the final preparation and its total enrichment could not be determined.

Purification of a high molecular weight PLA_2

The high molecular weight PLA_2 was purified from murine mammary carcinomas. Tumors were induced in syngeneic animals by injection of cells which had been previously demonstrated to have high levels of a Ca^{2+} translocatable PLA_2 [39]. Tumor tissue was homogenized in a buffer containing EDTA so that the PLA_2 of interest would be in the soluble fraction. The enzyme activity in the supernatant fraction following ammonium sulfate precipitation and high speed centrifugation was purified by sequential hydrophobic (Fig. 2A), anion exchange (Fig. 2B), hydroxyapatite (Fig. 2C) and gel filtration (Fig. 2D) chromatography. Table I

TABLE I

Purification of the high molecular weight PLA_2 from mouse mammary carcinomas

Purification step	Specific activity (nmol/mg per min) [a]	Purification (fold)
Homogenate (before $(NH_4)_2SO_4$)	0.15	1
Chromatography:		
hydrophobic	27	180
anion exchange	48	320
hydroxyapatite	915	6100
gel filtration	4432	22880

[a] nmol of radiolabeled arachidonic acid released per mg protein per min. Final recovery of activity was approx. 6% of the total starting activity.

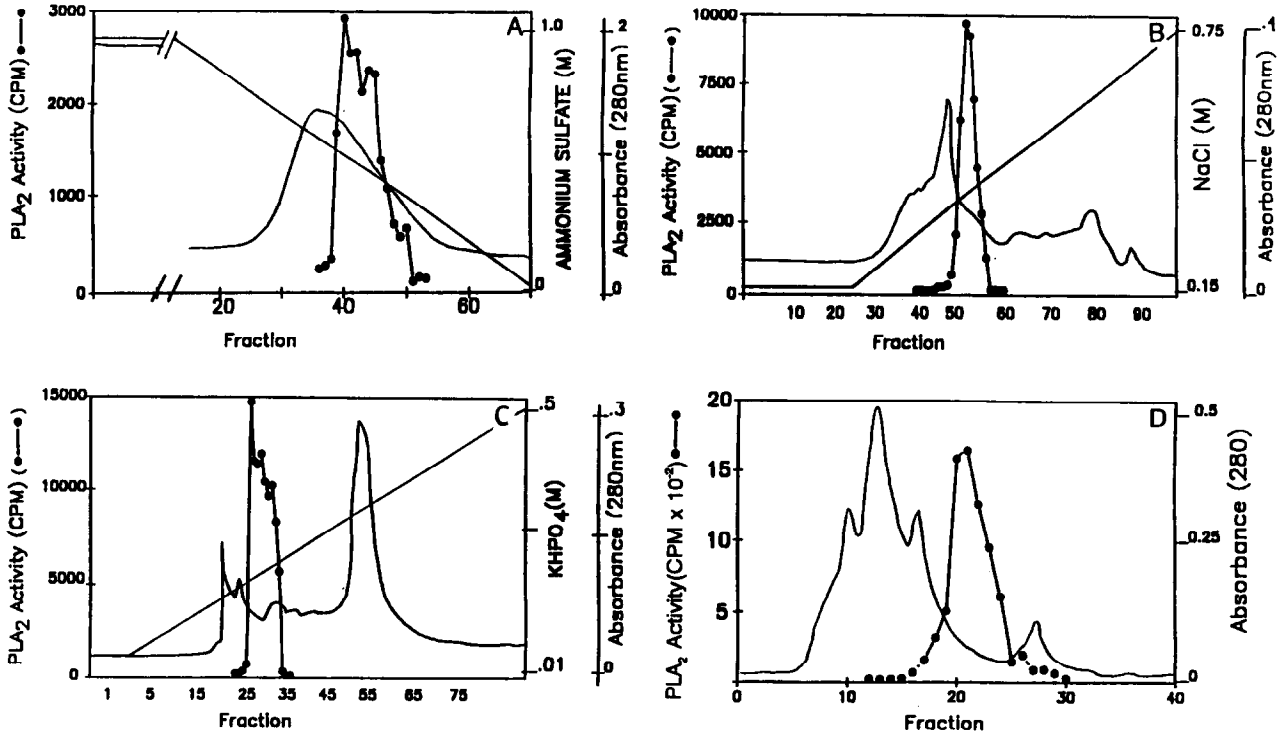

Fig. 2. Purification of a high molecular weight PLA_2. The high molecular weight PLA_2 was purified from mammary tumors by ammonium sulfate precipitation, high speed centrifugation, hydrophobic interaction (A), anion exchange (B), hydroxyapatite (C) and gel filtration (D) chromatography. PLA_2 activity (●) is expressed as counts per min of [^{14}C]arachidonic acid released per 2 min incubation per aliquot analyzed; ultraviolet absorbance and concentration of salt, M (------).

summarizes the purification of the high molecular weight PLA_2. The purification protocol was facilitated by the concentration of samples by ultrafiltration with membranes with a molecular weight cut-off of 30 kDa coupled with the use of a gel filtration system in which the PLA_2 had an anomalously low molecular weight.

Polyacrylamide gel electrophoresis of high and low molecular weight PLA_2s

To assess the purification of the low molecular weight PLA_2, fractions 8 to 11 from the immunoaffinity chromatography were pooled and an aliquot was radiolabeled using chloramine T and ^{125}I and analyzed by polyacrylamide gel electrophoresis. A single radiolabeled band was observed with a molecular weight of approx. 15 kDa (Fig. 3A). When the purified high molecular weight PLA_2 was analyzed by polyacrylamide gel electrophoresis/silver staining, a single band with a molecular weight greater than 100 kDa was observed (Fig. 3B).

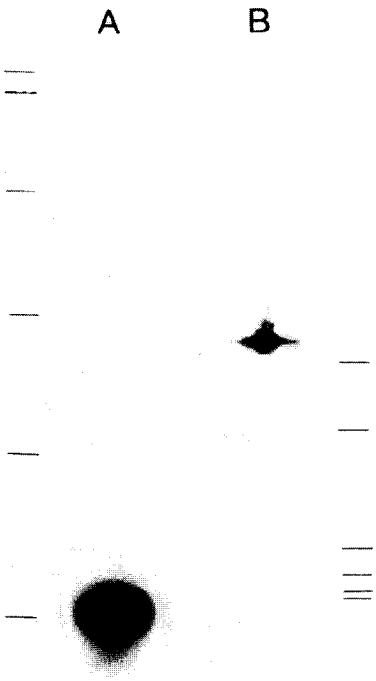

Fig. 3. Polyacrylamide gel electrophoresis of the low molecular weight PLA_2 (A) and the high molecular weight PLA_2 (B). (A) An aliquot of the pool of fractions 8 to 11 from the immunoaffinity HPLC of the low molecular weight PLA_2 was iodinated and then separated by polyacrylamide gel electrophoresis (15% gel) and radioactivity detected by radioautography; (B) The high molecular weight PLA_2 preparation, following gel filtration chromatography, was analyzed by polyacrylamide gel electrophoresis (7.5% gel)/silver staining. The horizontal barson the sides of the figure indicate the positions of the molecular weight standerds which were 98, 67, 43, 28, 19 and 14 kDa.

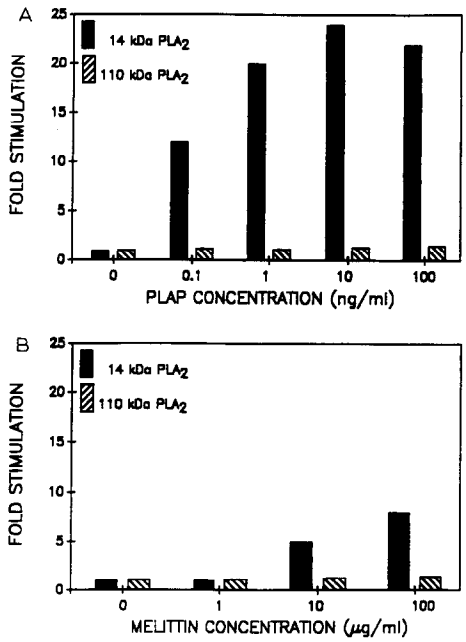

Fig. 4. Effects of PLAP (A) and melittin (B) on the activity of the low and high molecular weight PLA_2s. Fold stimulation is the activity observed in the presence of PLAP or melittin divided by the activity observed in the absence of PLAP and melittin. PLAP was purified from the BC_3H1 cell line as previously described [34], synthetic melittin was obtained from Peninsula (Belmont, CA). Enzyme activity was measured using 1-steroyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine at a concentration of 10 and 8 μM for the low and high molecular weight enzymes respectively. The approximate molecular weights of PLAP was 28000 and melittin was 2800.

Effect of PLAP and melittin on PLA_2 activity

With the purified PLA_2s in hand, the two enzymes were evaluated and compared with respect to potential enhancement of activity by PLAP and melittin. Incubation of the low molecular weight PLA_2 with increasing amounts of PLAP, resulted in a dose dependent increase in enzyme activity (Fig. 4A). In contrast, the activity of the high molecular weight PLA_2 was not affected by the addition of PLAP, when assayed under the same conditions (Fig. 4A). The effect of PLAP on the activity of the high molecular weight enzyme was also examined using except for the change in substrate to 8 μM 1-steroyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine. PLAP did not stimulate activity under these conditions or under suboptimal assay conditions in which the calcium was omited. Furthermore melittin, a PLA_2 activator, stimulated the activity of the low molecular weight PLA_2 while it did not the increase activity of the high molecular weight PLA_2 (Fig. 4B) under any of the reaction conditions mentioned above.

Discussion

Rapid, simple methods for purifying a high and low molecular weight PLA_2 are described. The two enzymes were examined for their responsiveness to PLAP

and to melittin. Stimulation of only the low molecular weight enzyme activity was observed. Thus, if PLAP stimulates the high molecular weight PLA_2, the assayes employed in this study are not suitable foe the detection of such stimulation. In view of the requirement for less than micromolar Ca^{2+} for activity and translocation of the high molecular weight enzyme, regulation of the high molecular weight enzyme might be mediated by regulation of intracellular Ca^{2+} concentration. In contrast, the low molecular weight enzyme, which on the basis of its immunoaffinity purification is likely to be a group II enzyme, is a candidate for regulation by PLAP. PLAP was more potent than melittin in the stimulation of the low molecular weight PLA_2, both with respect to the fold-stimulation and the concentration of protein/peptide necessary to observe stimulation. The molar ratio of melittin to phospholipid substrate is 1:3 for the melittin concentration of 10 μg/ml, i.e., a concentration at which melittin mediated stimulation of PLA_2 activity was observed. This is consistent with the proposed involvement of melittin interaction with phospholipid in the stimulation of PLA_2 activity (reviewed in Ref. 43). The molar ratio of PLAP to phospholipid was $1:10^6$ at a PLAP concentration of 0.1 ng/ml, where stimulation of PLA_2 activity was observed. This would suggest that PLAP mediated stimulation of PLA_2 activity might involve interaction of PLAP with either the enzyme alone or both enzyme and phospholipid. The latter possibility might involve the interaction of PLAP and phospholipid via the domain of PLAP with homology to melittin.

PLAP and the PLA_2 stimulating protein, bactericidal permeability increasing protein or BPI, have some interesting common features although they have distinctive amino acid sequences predicated from their cDNAs [37,44]. Both are positively charged, amphipathic proteins and are specific with respect to the PLA_2s they stimulate [37,45]. All the PLA_2s observed to be stimulated by BPI are positively charged PLA_2s although not all basic PLA_2s are stimulated by BPI (reviewed in Ref. 45). Thus far, the only PLA_2s which have been observed to be stimulated by PLAP are also positively charged, i.e., PLAP stimulated the low molecular weight PLA_2 which bound to the cation exchange column, but not the high molecular weight PLA_2 which bound to the anion exchange column. Neither BPI [45] nor PLAP [cited in 34] stimulated pancreatic PLA_2 activity thus distinguishing them from PLA_2 activating proteins characterized from rat brain [46] and calf thymocytes [47] which do stimulate the pancreatic enzyme. The combined findings suggest that PLA_2 activating proteins are specific with respect to the particular enzyme(s) they stimulate and hence may provide specificity in the regulation of various PLA_2 activities.

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