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ISOLATION OF HOMOGENEOUS PHOSPHOLIPASE A_2 FROM HUMAN PLATELETS

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Summary: Phospholipase A_2 from human blood platelets has been purified to homogeneity by treatment of the platelet homogenate with H_2SO_4, followed by affinity chromatography of the extract. The platelet phospholipase is a soluble, heat-stable protein of MW 44000. The purified enzyme inhibits ADP-induced platelet aggregation.

Introduction. The phospholipase A_2 activity in blood platelets has been implicated as a key step for the synthesis of thromboxane A_2(TXA_2) and prostaglandins from the arachidonic acid liberated *in situ* from membrane phospholipids (1,2). Also a congenital deficiency of phospholipase A_2 activity has been observed in a variant of Hermansky-Pudlak syndrome, in which platelet aggregation is impaired (3). To better understand the putative role of phospholipase A_2 and its regulation, we have purified and partially characterized this enzyme from human blood platelets.

Materials and Methods. The following published procedures were used for obtaining the materials used in this study. Egg phosphatidyl-choline was prepared from hen egg yolks (4). The labelled substrate. 2-[^{14}C]linolinoyl-phosphatidylcholine, was prepared by acylation of lysophosphatidylcholine (obtained by hydrolysis of egg phosphatidylcholine by phospholipase A_2 from *Naja naja* venom) with rat liver homogenate (5). The specific activity of the product was ≃1 mCi/mmol and all (>98%) the label was released in the fatty acid fraction by phospholipase A_2 from *Naja naja* or bee venoms. The lecithin analog, rac-1-octa-dec-9-enyl-2-hexadecylglycero-3-phosphocholine (Calbiochem) was oxidized with potassium periodate and potassium permanganate (6,7) to yield rac-1-(9-carboxy)nonyl-2-hexadecylglycero-3-

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phosphocholine, which was coupled to AH-Sepharose 4B (Sigma) to yield the affinity adsorbant (8). The phosphorus content of the affinity gel was ≃2.5 µmol/ml of the swollen and packed gel. Platelet rich plasma from freshly drawn human blood was obtained as described elsewhere (9).

Phospholipase A_2 activity was measured by either of the following two procedures: (a) The radiolabelled substrate dissolved in wet diethylether was incubated with the enzyme (10), and the products were separated by thin layer chromatography on silica gel plates (Merck) in chloroform + methanol + water (65:35:6), where the fatty acid, diacylphosphatidylcholine and lysophosphatidylcholine migrate at different rates (R_f ≃ 0.95, 0.33 and 0.10 respectively. The radioactivity in appropriate spots was visualized with a Beta-Camera LB 290 A (Berthold, Germany) which gave a photographic record. For quantitation of the radioactivity in the spots, the appropriate regions on the t.l.c. plate, after extracting the scraped silica gel were counted. The substrate and products could also be separated by column chromatography on neutral alumina (Woelm) where fatty acid is eluted by chloroform, phosphatidylcholine by chloroform + methanol (7:3), and lysophosphatidylcholine by methanol. (b) Phospholipase A_2 activity was routinely measured titrimetrically as described elsewhere (11, 12). Unless stated otherwise the titrations were carried out with 2.5 mM sodium hydroxide at 37°C, pH 9.5 in aqueous solution containing 100 mM KCl, 20 mM $CaCl_2$ and 500 µM egg phosphatidylcholine as unilamellar vesicles obtained by sociation in a bath type sonicator (Heat Systems). Control experiments demonstrated that under these conditions there is no detectable proteolytic activity as measured with Azoalbumin (Sigma) (13) even in the whole platelet homogenate. With appropriate precautions we could routinely measure rates of proton release < nmol/min.

Results: The overall purification of phospholipase A_2 from platelets was essentially achieved in two steps. Typically, platelets from 200 ml of platelet rich plasma were separated on a 10-40% linear glycerol gradient (9, 14) and homogenized in distilled water with an all glass Kontes type B homogenizer. The homogenate was made up to 0.16 N H_2SO_4 in 40 ml total volume (15). After 30 minutes the homogenate was centrifuged (20 min, 23000 g) and the supernatant was extensively (36 hrs) dialyzed with several changes of 1 mM Tris, pH 7.5, and finally against distilled water. The white precipitate in the dialysis bag was removed by centrifugation, and the supernatant was lyophilized. The residue was dissolved in solution I (20 mM $CaCl_2$ and 100 mM KCl) and applied to the affinity column (40 ml packed swollen

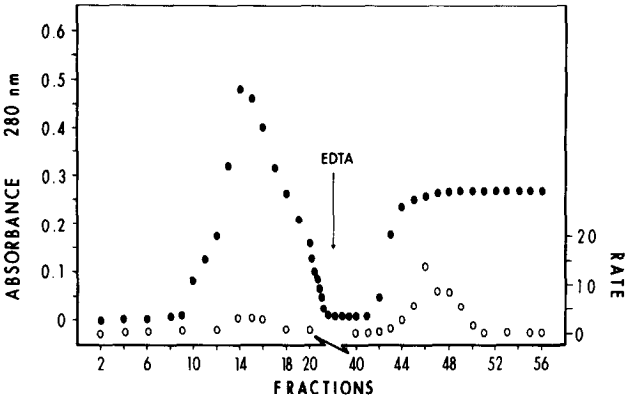

Fig. 1.- Elution profile obtained from the affinity column obtained as described in Methods. In this particular experiment, 1.2 ml fractions were collected. Rate is arbitrarily expressed as divisions per minute.

volume) pre-equilibrated in the same solution. The column was washed with solution I till the effluent showed no absorption at 280 nm. The adsorbed phospholipase A activity was eluted with the moving front of the solution II (40 mM EDTA, 100 mM KCl, pH 8.0). A typical elution profile for the protein (A_{280}) and for the phospholipase A activity is presented in Fig. 1.

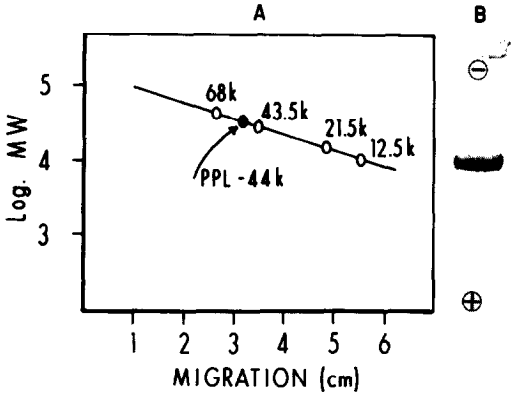

Fig. 2.- SDS-polyacrylamide pattern of purified platelet phospholipase (PPL) in a 4-40% acrylamide gradient. 15 µgr. protein were loaded on the gel (right). Comparative migration of PPL with regard to known MW protein standards (4-40% acrylamide): cytochrome C (12.5 K), soybean trypsin inhibitor (21.5 K), ovalbumin (43.5 K), and bovine serum albumin (68 K).

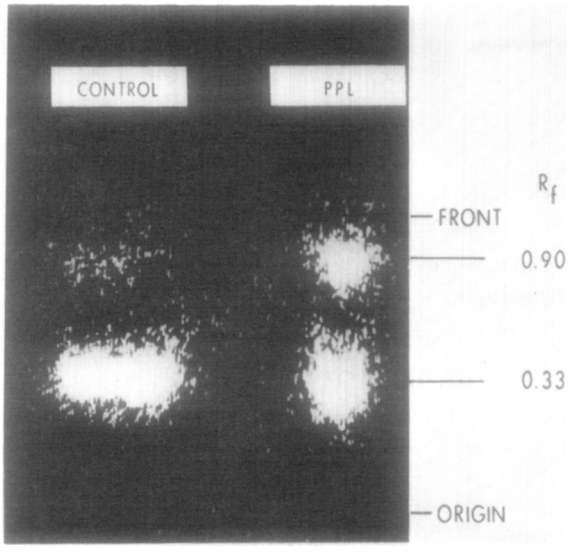

Fig. 3.- Hydrolysis of C-2 radiolabelled phosphatidylcholine by purified platelet phospholipase. Reaction was performed in diethylether and the products separated by thin layer chromatography, as described in Methods. In this system, Rf for fatty acid is 0.87, for phosphatidylcholine is 0.35 and lysophosphatidylcholine do not migrate from the origin (16).

The fractions containing the enzymatic activity were pooled, dialyzed extensively against water, and then lyophilized. On a 4-40% gradient polyacrylamide gel in SDS, the purified protein showed a single band, both in the presence and in the absence of sodium dodecylsulfate (Fig. 2). The estimated molecular weight was 44000 dalton, which coincides with the behaviour of the enzyme in gel filtration. A minor band of higher MW, constituting less than 1% of the total purified protein, was sometimes seen when SDS was excluded.

The rate of hydrolysis of phosphatidylcholine by phospholipase A_2 is enhanced in the presence of additives like diethylether, certain detergents and n-hexanol. The labelled 2-linolenoylphosphatidylcholine dissolved in diethylether releases the fatty acid in the 2-position, and no label is found in the lysophosphatidyl-

choline fraction (Fig. 3). This suggests that the enzyme we have isolated is exclusively phospholipase A_2, however, both phospholipase A_1 and A_2 activities could be identified in homogenized platelets (3).

As shown in Table I the overall purification of the phospholipase from homogenized platelets was > 3000 fold with ≃ 40% overall recovery of the activity. The overall purification from the platelet rich plasma is over 500,000 fold. The specific activity of the lyophillized protein is 530 $nmol \cdot mg^{-1} \cdot min^{-1}$ with sonicated egg phosphatidylcholine containing 45 mM n-hexanol and 10 mM $CaCl_2$. The enzymic activity is found to be quite sensitive to the nature of the lipid and the activator (to be published). Under these conditions the rate of hydrolysis of the substrate varies linearly with te protein concentration. The optimum pH for the enzyme is at 9.4-9.5. It has an absolute requirement for Ca^{2+}, and the maximal enzymatic activity is achieved at ≃ 5 mM Ca^{2+}. Similar results have been obtained by Franson et al. (16) with a partially purified preparation. The rate of hydrolysis was found to be independent of the sequence of addition of the enzyme, substrate, cofactor (Ca^{2+}), or the activator (n-hexanol).

Table I. Phospholipase Activity of Human Platelets after Various Purification Steps.

Treatment	Protein (% of Initial)	Phospholipase Activity* % of Initial	Phospholipase Activity* $nmol \cdot min^{-1} \cdot mg^{-1}$
None	100	100	0.4
1.6 N H_2SO_4 and dialysis	10	58	2.3
Affinity column eluate	0.03	40	530

*Activity is measured with unilamellar egg phosphatidylcholine vesicles in the presence of 45 mM n-hexanol at pH 9.5.

Discussion. In the preceding section we have outlined the first procedure for a complete purification of a phospholipase A_2 from human platelets. The molecular weight of the purified enzyme (44000 dalton) differs from that reported for the enzyme obtained from polymorphonuclear leukocytes (< 25000) (13) or from sheep erythrocyte membranes (18500) (8). Like the enzyme from other sources, it is a soluble protein, it acts on phospholipid bilayers modified by n-hexanol, on the substrate dissolved in diethyl-ether, or on the substrate dispersed as micelles in detergents. For the hexanol-modified egg-phosphatidylcholine bilayers the specific activity of the platelet enzyme is about 1000 fold lower than that for the bee venom, snake venom or pig pancreatic enzymes measured under identical conditions (11,12). In contrast, compared to the other enzymes, the platelet enzyme is several hundred fold more effective in inhibiting ADP-induced platelet aggregation. We suspect that this is due to a difference in the substrate specificity, and due to a difference in the state in which the substrate is presented to the enzyme. Studies with the purified platelet phospholipase A_2 may help to resolve these and other possibilities.

Physiologically, the action of endogenous phospholipase A_2 has been implicated in platelet aggregation (17,18). The effect of platelet phospholipase on platelet aggregation is shown in Fig. 4. The data shows that the platelet enzyme ($\simeq$ 3 µg or 0.0015 units) inhibits the second wave of ADP-induced aggregation. The second wave of aggregation is inhibited by preincubating the platelets with $\simeq$ 0.0015 units of the enzyme for less than one minute. Longer (> 3 minutes) periods of incubation block the first wave of aggregation. which is also blocked by larger amounts of the enzyme. In contrast, arachidonic acid in-

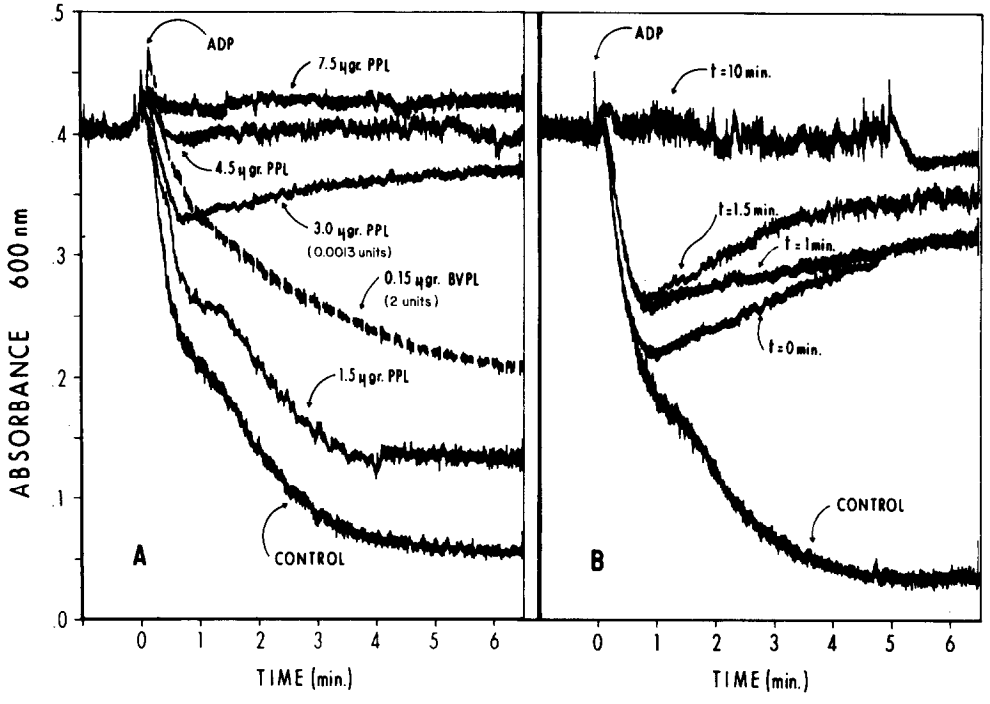

Fig. 4.- The effect of purified platelet phospholipase on ADP-induced platelet aggregation (7 μM ADP) at 37°C. A) Concentration dependence, B) Time dependence. Bee venom phospholipase (BVPL) has been included for comparison.

duces platelet aggregation at > 200 μM (17). Moreover, the amount of enzyme is such that even under optimal conditions this much fatty acid could not be released, and most of the common naturally occurring fatty acids do not inhibit ADP-induced aggregation (17). As suggested elsewhere (19,20) the inhibitory effect of phospholipase A_2 treatment on platelet aggregation is most likely due to a modification of the outer surface of the platelets. Interestingly the snake venom (18,19), pig pancreatic (20) and bee venom phospholipase A_2 (Fig. 4) induce the inhibitory effect at much higher concentrations (> 2 units). These observations would imply that phospholipids on the outer surface of platelets are much more susceptible to the platelet phospholipase A_2 than to the enzyme from other sources. In

our experiments, besides the action of the enzyme only on the outer surface, other factors arising from substrate specificity of the enzyme and also from the organization of the substrate in the membrane, could contribute to this particular behaviour (21). Indeed, preliminary experiments suggest that depending upon the substrate and the activator used, the rate of hydrolysis catalyzed by the platelet enzyme changes at least several hundred fold.

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