Phospholipase A₁ of Human Blood Platelets

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Substantial phospholipase A₁ activity has been demonstrated in human blood platelets, and a rapid method for its measurement is described. The enzyme requires taurocholate for full activity and in these conditions the pH optimum is 4.8. The phospholipase activity is released from platelets by incubation with thrombin.

Phospholipase A (EC 3.1.1.4) with acid pH optimum has been shown to be present in lysosomes of adrenal medulla, liver and macrophages (Smith & Winkler, 1968; Stoffel & Trabert, 1969; Waite *et al.*, 1969; Franson *et al.*, 1971, 1972). Since the specific activity of acid hydrolases in platelets is high (Day *et al.*, 1969), we looked for phospholipase A activity at acid pH in various platelet preparations. Phospholipase A is now recognized to include phospholipases A₁ and A₂, which respectively hydrolyse ester linkages at the C-1 and C-2 positions (Lennarz, 1970). These enzymes can be distinguished by characterizing the lysophospholipid that is formed. We examined platelets for both types of activity and studied phospholipase A₁ activity by using a new rapid method.

Methods and materials

Platelet preparations. Human venous blood (170 ml) was collected in a silicone-treated glass bottle containing 10 ml of 100 mM-EDTA. The blood was centrifuged at 900g for 15 min at room temperature to prepare platelet-rich plasma. This was further centrifuged at 2400g for 10 min at 4°C to sediment platelets. The supernatant plasma was removed and the platelets were washed in a buffered saline solution [134 mM-NaCl – 15 mM-Tris – HCl buffer (pH 7.4) – 5 mM-glucose – 1 mM-EDTA]. The platelets were then suspended in a small volume of the same buffer without EDTA so that there were 3×10^9 – 10×10^9 platelets/ml, i.e. 6–20 mg of protein/ml. A portion of this platelet suspension was taken for the determination of total phospholipase A₁ activity in the platelets. Other enzyme preparations were made by incubating platelet suspensions at 37°C for 5 min with 5 units of thrombin (bovine; Parke Davis, Pontypool, Mon., U.K.)/ml and centrifuging to yield a 'lysosomal'

preparation and a platelet pellet (Holmsen & Day, 1970). The pellet was resuspended in the original volume of buffered saline without EDTA. Protein was determined by the method of Gornall *et al.* (1949).

Labelled substrates. ([1-³H]Stearoyl) phosphatidylcholine was prepared by incubating [9,10-³H]-stearic acid (0.12 μmol; 5 mCi; The Radiochemical Centre, Amersham, Bucks., U.K.), linoleic acid (1 μmol) (to block incorporation of stearic acid into the 2-position), ATP (20 μmol), CoA (0.20 μmol), MgCl₂ (10 μmol) and NaF (310 μmol) with a rat liver homogenate (0.2 g in 1 ml of water). ([2-¹⁴C]Linoleoyl) phosphatidylcholine was prepared in similar fashion except that [1-¹⁴C]linoleic acid (3 μmol; 10 μCi; The Radiochemical Centre) and 1-acyl-lysophosphatidylcholine (3 μmol) were used as substrates. The final volume was 2.3 ml, incubation was for 2 h at 37°C and the pH was 7.4 (40 mM-potassium phosphate buffer). The labelled phospholipids were extracted (Bligh & Dyer, 1959) and purified by column chromatography on silicic acid. Pure phosphatidylcholine was eluted with CHCl₃–methanol mixture (4:1 v/v) containing 1.25% (v/v) of water (Webster & Thompson, 1962) and stored in CHCl₃ at –20°C. To determine the intramolecular distribution of labelling, samples were treated with snake venom (*Agkistrodon piscivorus piscivorus*; Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A.) to hydrolyse the ester bond at the 2-position, and the radioactivity in the free fatty acid and the lyso derivative formed was measured: 96% of the radioactivity in ([1-³H]stearoyl) phosphatidylcholine was recovered in the lyso derivative and 96% of the radioactivity in ([2-¹⁴C]linoleoyl) phosphatidylcholine was recovered in the free fatty acid.

Phospholipase A₁ assay. ([1-³H]Stearoyl) phosphatidylcholine (20000 c.p.m.; 250 nmol) in 10 μl of CHCl₃ was deposited at the bottom of a 12 ml conical-

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bottomed tube. The CHCl_3 was evaporated off and the phospholipid substrate was solubilized in $50\mu\text{l}$ of sodium taurocholate solution buffered with 0.2M -acetate ($\text{pH } 3.5\text{--}5.5$) or 0.2M -imidazole-HCl ($\text{pH } 6.0\text{--}8.0$). The reaction was started by adding $50\mu\text{l}$ of enzyme preparation and stopped by adding 2.0ml of di-isopropyl ether-methanol ($99:1$, v/v) and 150mg of silicic acid (200 mesh). Immediately the tube was agitated on a Vortex mixer for 10s , allowed to stand at room temperature for 10min , re-agitated for 10s and then centrifuged at $400g$ for 1min . A 1.0ml portion of the supernatant solution, which contained all of the liberated fatty acid and almost no phospholipid ($<1\%$), was transferred to a counting vial, 10ml of scintillation fluid (Buhler, 1962) was added and the radioactivity of the liberated fatty acid was determined in a Packard 3375 liquid-scintillation spectrometer.

In every experiment controls were included for non-enzymic hydrolysis of phosphatidylcholine and for recovery of added radioactive stearic acid (8000c.p.m.).

Results

We demonstrated and then characterized phospholipase A₁ in platelets as follows. A platelet suspension was incubated at 37°C with 2.5mM -([$1\text{-}^3\text{H}$]stearoyl) phosphatidylcholine or ([$2\text{-}^{14}\text{C}$]linoleoyl) phosphatidylcholine solubilized in sodium taurocholate (6mg/ml) at $\text{pH } 4.5$. The contents of the reaction tubes were extracted and the extracts subjected to t.l.c. (Skidmore & Entenman, 1962). Radioactivity in the fatty acid, lysophosphatidylcholine, phosphatidylcholine and all other zones was determined (Shaw & Harlan, 1971). When incubation tubes contained ([$1\text{-}^3\text{H}$]stearoyl) phosphatidylcholine there was a decrease in phosphatidylcholine that was totally accounted for by an increase in fatty acid. When incubation tubes contained ([$2\text{-}^{14}\text{C}$]linoleoyl) phosphatidylcholine 84% of the decrease in phosphatidylcholine was accounted for by an increase in labelled lysophosphatidylcholine (due to phospholipase A₁) and 16% by an increase in radioactive free fatty acid (presumably due to lysophospholipase acting after phospholipase A₁). These results indicated that platelets contain principally phospholipase A₁ activity at acid pH. Thenceforth this activity was determined by measuring the release of [^3H]stearic acid from ([$1\text{-}^3\text{H}$]stearoyl) phosphatidylcholine as described above under 'Methods and materials'.

Early in the course of the studies we noted that phospholipase A₁ activity at acid pH was dependent on the concentration of sodium taurocholate. Fig. 1 shows the effects of various concentrations of taurocholate on the phospholipase activity of two of the platelet preparations. Little or no activity was found in the absence of taurocholate. The platelet suspension

Fig. 1. Effects of sodium taurocholate on the phospholipase A₁ activity at $\text{pH } 4.5$ of two platelet preparations

The reaction was started by adding platelet suspension ($\circ$) containing $600\mu\text{g}$ of protein or 'lysosomal' preparation ($\bullet$) containing $50\mu\text{g}$ of protein. Incubation was for 3h at 37°C . Other details are described in the text under 'Methods and materials'.

showed highest activity at 6mg of taurocholate/ml, whereas the 'lysosomal' preparation had highest activity at 3mg/ml .

Optimal activity for both the platelet suspensions and the 'lysosomal' preparations was found at $\text{pH } 4.8$. Activity was not changed significantly by ultrasonication, freezing and thawing, 5mM-Ca^{2+} or 5mM-EDTA . Heating at 60°C for 5min destroyed 18% of the activity of a platelet suspension and 32% of that of a 'lysosomal' preparation. Heating at 100°C for 5min destroyed 99% of the activity of both preparations. The platelet suspensions used for this work contained small numbers of erythrocytes (0.2% or $<1 \times 10^5/\text{incubation tube}$). These cells did not contribute to the measured phospholipase A₁ activity of the platelets, because enzyme studies with 3×10^9 washed erythrocytes/incubation tube revealed no activity. The thrombin used to prepare the 'lysosomal' preparations contained no phospholipase A₁ activity.

The liberation of [^3H]stearic acid was measured as a function of time with platelet suspensions and 'lysosomal' preparations. The platelet suspensions showed high activity during the first hour and then hydrolysis continued linearly at a somewhat diminished rate. Phosphatidylcholine hydrolysis by the 'lysosomal' preparations was almost linear with respect to time. With various amounts of 'lysosomal' preparations activity was linear up to $70\mu\text{g}$ of protein/incubation tube. However, when similar experiments were carried out with different amounts of platelet

Fig. 2. Phospholipase A₁ activity of different amounts of platelet suspension as a function of sodium taurocholate concentration (a) or protein (b)

The reaction was started by adding platelet suspension (150–600 μg of protein). Incubation was for 3 h at 37°C. Other details are described in the text under 'Methods and materials'. The curves in (a) were obtained with the following amounts of protein: ○, 150 μg; ●, 300 μg; △, 450 μg; ▲, 600 μg. Optimal activities in (a) were used to plot the curve shown in (b) with the following sodium taurocholate concentrations: ○, 4 mg/ml; ●, 5 mg/ml; △, 6 mg/ml; ▲, 7 mg/ml.

suspensions activity was linear only when taurocholate concentrations were optimal (Fig. 2).

To assess the proportion of phospholipase A₁ that thrombin released from platelets, the activity in 'lysosomal' preparations was compared with that in the mother platelet suspensions and pellet preparations. Each preparation was evaluated at its optimal taurocholate concentration. The mean results of three experiments (three donors) suggested that about 50% of the phospholipase A₁ in platelets was released by thrombin.

Discussion

The formation of radioactive lysophosphatidylcholine from ([2-¹⁴C]linoleoyl) phosphatidylcholine and the release of radioactive fatty acid from ([1-³H]-stearoyl) phosphatidylcholine demonstrated that human platelets contain phospholipase A₁ activity. The detection of this activity at acid pH supported our belief that the enzyme was of lysosomal origin.

Thrombin is known to release several lysosomal enzymes from platelets, but does not release enzymes associated with mitochondria, cytoplasm or membranes (Holmsen & Day, 1970). This 'release reaction' results in considerable purification of the acyl hydrolases of platelets because the bulk of the platelet proteins and lipids are not released (Davey & Lüscher, 1968). Therefore we used the supernatant from throm-

bin-treated platelet suspensions as our 'lysosomal' preparations.

Comparison of the characteristics of the phospholipase A₁ activity in the 'lysosomal' preparations with those in platelet suspensions revealed one main difference. More taurocholate was required to demonstrate maximal activity when the platelet suspensions were studied. This may be related to the large amounts of endogenous lipid (Barkhan *et al.*, 1961) or protein present in platelets but not in the lysosomal preparations (Davey & Lüscher, 1968).

Platelet suspensions could be 'titrated' with sodium taurocholate to reveal maximal activity, and we made the assumption that this maximal activity represented the total activity in platelet suspensions. In this way we were able to estimate that about 50% of this enzyme was released from platelets by thrombin. This is similar to the proportions of β-N-acetylglucosaminidase, β-galactosidase and cathepsin released under similar conditions (Holmsen & Day, 1970).

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