Biochimica et Biophysica Acta, 792 (1984) 99–102
Elsevier

BBA Report

BBA 50057

MURINE ERYTHROCYTES CONTAIN HIGH LEVELS OF LYSOPHOSPHOLIPASE ACTIVITY

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(Received April 27th, 1983)
(Revised manuscript received October 19th, 1983)

Key words: Enzyme activity; Lysophospholipase; (Murine erythrocyte)

Murine erythrocytes were found to be unique in the high levels of lysophospholipase activity in the cytosol of these cells. The specific activity of the enzyme in the cytosol of the murine cells is 10-times higher than in the cytosol of rabbit erythrocytes and approximately three orders of magnitude higher than those in the red cells of rat, man, pig and ox.

Lysophospholipases (EC 3.1.1.5) occur often in nature (see Ref. 1 for a recent review) and are believed to play an essential role in preventing the accumulation of lysophosphoglycerides which are known to perturb cellular membranes [2]. The presence of these enzymes has been demonstrated in erythrocytes, although at relatively low levels. Heemskerk and Van Deenen [3] observed that the treatment of haemolysates of rabbit erythrocytes with *Crotalus adamanteus* venom, known to contain phospholipase A_2 (EC 3.1.1.4) activity, did not result in the production of lyso-compounds. Chromatographic analyses of the water-soluble fraction of these reaction mixtures revealed substantial amounts of glycerophosphorylcholine and glycerophosphorylethanolamine. The authors concluded that the lyso-derivatives, produced by phospholipase A_2-catalyzed degradation of membrane phosphatidylcholine and phosphatidylethanolamine, had been degraded further by a lysophospholipase present in the haemolysates. The addition of exogenous lysophosphatidylcholine to haemolysates of human [4,5], bovine [5] or rabbit [5] erythrocytes, under conditions not favouring its reacylation to diacyl-phosphatidylcholine, also appeared to result in the production of glycerophosphorylcholine. The haemolysates from rabbit erythrocytes appeared to be the most active ones in this respect.

During our studies on (murine) Friend erythroleukaemic cells, we recently observed that extensive treatment of disrupted cells with pure *Naja naja* phospholipase A_2 did not produce lysophospholipids, although all diacylglycerophospholipids had been degraded to completion (Rogers, J.A., Op den Kamp, J.A.F. and Roelofsen, B., unpublished observations). Subsequent studies revealed the presence of lysophospholipase activity in the cytosol of these cells. Since Friend cells are believed to represent genuine (pro)erythroblasts [6], one of the earliest stages in the development ultimately leading to the mature red cell, we thought that the high lysophospholipase activity may be specific for the erythroblasts and may play an essential role in phospholipid metabolism during this, and subsequent, stages of erythrocyte biogenesis. This prompted us to determine the level of lysophospholipase activity in the cytosol of mature murine erythrocytes and, as this level was found to be unexpectedly high, to compare this with the levels of lysophospholipase activity in the cytosols of mature erythrocytes from a number of other mammalian species.

Blood was collected from mice and rats following decapitation and from rabbits and human volunteers by venepuncture. Porcine and bovine blood was obtained from the local slaughterhouse. Standard acid/citrate/dextrose was used as anticoagulant in all cases. After centrifugation for 10 min at 2500 × *g* and removal of serum and buffy coat, the erythrocytes were washed at least three times with 5 vol. of isotonic saline. Washed erythrocytes were lysed either by three cycles of freezing and thawing using a solid CO_2/acetone mixture, or by osmotic shock in 10 vol. of 10 mM phosphate buffer, containing 0.1 mM EDTA, pH 7.4. Ghosts were removed by centrifugation for 20 min at 30 000 × *g* (Sorvall, SS-34 rotor). Lysophospholipase activity was determined as described in the legend to Table I, essentially following the procedure of Van den Bosch et al. [7].

As can be seen from Table I, the cytosol of murine erythrocytes has a high lysophospholipase activity. Although the presence of this enzyme was also detected in the cytosol of human, bovine, porcine and rat erythrocytes, their specific activities were almost three orders of magnitude lower than that in the cytosol of the erythrocytes from mice (Table I). In agreement with earlier studies [3,4], considerable levels of lysophospholipase activity were also found in rabbit erythrocytes, but, although the total amount of activity in this case was 25–50-times higher than that in the other four mammalian species, it still was only 10% of that found in mice (Table I).

Like the enzyme from other sources [1], the lysophospholipase from murine erythrocytes does not require divalent cations for activity, since no inhibition was observed when the assay was carried out in the presence of 5 mM EDTA. Similarly, addition of 5 mM $CaCl_2$ to the incubation mixture did not have any effect.

Preliminary attempts to purify to homogeneity the lysophospholipase from murine erythrocytes were hampered by the fact that the enzyme appeared to be highly unstable at pH values outside the physiological range (7.0–7.5) and the limited quantity of starting material due to the very small volumes of blood which can be derived from these animals. The highest purification factor we were able to obtain was achieved by two passes of the diluted cytosol over a DEAE-cellulose ion-exchange column, operated at pH 7.15 and using NaCl gradients. This two-step purification resulted in a preparation with a specific activity of 2.1 U/mg protein. Although this value is similar to that reported for homogeneous preparations of lysophospholipases purified from other sources (bovine liver enzymes I and II, 1.5 U/mg [11]; rat lung, 2.1 U/mg [12]; *Escherichia coli*, 2.6 U/mg [13]), our preparation appeared to be contaminated with at least seven other proteins as judged from SDS-polyacrylamide gel electrophore-

TABLE I

OCCURRENCE OF LYSOPHOSPHOLIPASE ACTIVITY IN THE CYTOSOL OF ERYTHROCYTES OF VARIOUS MAMMALIAN SPECIES

1-Acyl-*sn*-glycero-3-phosphorylcholine was prepared by treating egg phosphatidylcholine with *C. adamanteus* phospholipase A_2 in ether/borate buffer, pH 7.0 [8]. It was subsequently purified by preparative thin-layer chromatography using silica gel G thin-layer plates and chloroform/methanol/conc. ammonia/water (90:54:5.5:5.5, v/v) as the developing system. This pure lysophosphatidylcholine was used to dilute 1[^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (a generous gift from Dr. A.J. Aarsman) to a specific radioactivity of 100000 dpm/μmol. 100 nmol of this ^{14}C-labeled substrate were incubated in 0.5 ml 20 mM phosphate buffer (pH 7.0) with samples of the cytosols, prepared as described in the text, containing appropriate amounts of protein. After incubation for 7 min at 37°C, the reaction was stopped by the addition of 2.5 ml isopropanol/*n*-heptane/0.5 M H_2SO_4 (40:10:1, v/v), the fatty acids partitioning in the *n*-heptane layer [9]. A 1-ml sample of the heptane layer was pipetted into a scintillation vial containing 10 ml of toluene scintillation mixture (5 g 2,5-diphenyloxazole, 0.25 g 1,4-bis(2-(5-phenyloxazolyl))benzene and 50 ml Biosolv per liter toluene). Radioactivity was determined in either a Packard Tricarb Model 3003, or a Searle Isocap 300, scintillation spectrometer. 1 unit of lysophospholipase activity was defined as the amount of enzyme which degrades 1 μmol of substrate in 1 min at 37°C. The protein content of the preparations was determined by the method of Lowry et al. [10].

Mammalian species	Spec. act. [a] (mU/mg protein)	Relative spec. act. (%)
Mouse	10.85 ± 0.7 (6)	100.0
Rat	0.02 ± 0.01 (4)	0.2
Rabbit	1.14 ± 0.20 (5)	10.5
Man	0.03 ± 0.02 (5)	0.3
Pig	0.03 ± 0.01 (4)	0.3
Ox	0.04 ± 0.01 (4)	0.4

[a] Means ± S.D. Number of determinations in parentheses.

sis (results not shown). Hence, the real specific activity of the lysophospholipase will be considerably higher than 2.1 U/mg and may even be in the range of that of the lysophospholipase from *Vibrio parahaemolyticus*, 48 U/mg [14]. The latter is the highest specific activity reported so far for a homogeneous preparation of this enzyme.

A most intriguing question arises, concerning the physiological significance of the high levels of lysophospholipase activity which appeared to be specific for the erythrocytes from mice. As already mentioned, lysophospholipase activity has also been found in the cytosol of undifferentiated murine Friend erythroleukaemic cells (cell line GM-86, clone 745), which are considered to be pro-erythroblasts, one of the earliest precursors of the erythrocyte. The specific activity of the enzyme in the cytosol of these cells (2.4 mU/mg; Sanderink, G., unpublished observation), however, is only about 25% of that found in the cytosol of the mature red cell (Table I). Taking into account the fact that the total protein content of the cytosol of the Friend cells and that in the mature erythrocytes are approx. 100 mg/ml (Rawyler, A.J., personal communication) and 350 mg/ml, respectively, it follows that the mature erythrocyte contains 15-times more of the active enzyme than its precursor. Assuming that differences in the strains of mice involved in these studies (mature erythrocytes and Friend cells originated from BALB/c and DBA/2J mice, respectively) do not account for the above difference in lysophospholipase activity, the increase which apparently takes place during the differentiation process obviously points to an essential function of the enzyme in the mature erythrocyte. Since the occurrence of (lyso)phospholipids in the erythrocyte is confined to the plasma membrane of the cell, it seems plausible to suppose that a functional relationship might exist between the lysophospholipase and this membrane. Therefore, it is of interest to note that, although up to 80% of the total enzymatic activity was found in the supernatant when the membranes had been removed by high-speed centrifugation following freeze-thawing of the cells, no more than 50% of the total lysophospholipase activity could be found in such a supernatant after lysis of the cells by osmotic shock. However, one additional wash of the latter ghost pellet with hypotonic buffer was sufficient to solubilize 95% of the remaining activity. This may suggest that the enzyme indeed shows a tendency to maintain (weak) interactions with the plasma membrane, which in turn may support the above proposed functional relationship between the two. The most plausible function of the lysophospholipase seems to be that it provides the possibility of protecting the plasma membrane against harmful concentrations of lysophospholipids. This still leaves the intriguing question, however, as to why the murine erythrocyte is specifically equipped with such high levels of this enzyme. It is tempting to speculate that this particular erythrocyte species may be deficient in the enzyme system that catalyzes the (re)acylation of lysophospholipids, a system which has been convincingly demonstrated to be present in the plasma membrane of a number of other mammalian erythrocytes [4] and which appeared to be located at the cytoplasmic side of the human [15] and rat [16] erythrocyte membrane.

We are indebted to Dr. P. Child for critical reading of the manuscript.

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