CHARACTERIZATION OF A MONOESTER LIPASE ACTIVE AS MEMBRANE-BOUND ENZYME IN RAT ERYTHROCYTES

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1. Introduction

It has been reported [1] that upon treatment by phospholipase C of membranes from rat erythrocytes, little or no diacylglycerol accumulated in the preparations, suggesting that diacylglycerol derived from phospholipid hydrolysis had been degraded further by an endogenous diacylglycerol lipase. At present, however, there is no information on the number, nature and behavior of lipase(s) associated with the erythrocyte membrane.

This paper is concerned with the characterization of a monoester lipase measurable in vitro with whole intact erythrocytes from rats (and other species) as source of enzyme. The results suggest that this lipase acts as an enzyme firmly attached to the membrane, with its active site externally oriented. This lipase is very likely to be different from another monoester lipase present in plasma as a soluble enzyme.

2. Materials and methods

Blood was obtained by cardiac puncture from male Sprague-Dawley rats (220–250 g), using EDTA (1 mg/ml) as anticoagulant. The plasma was removed by centrifugation and erythrocytes were washed 3 times with 4 vol. 0.172 M Tris–HCl (pH 7.6). The buffer layer was discarded by careful suction on each occasion. Erythrocyte ghosts were prepared using hypotonic (20 mOsm/kg) Tris buffer [2].

Unless otherwise stated monoester lipase activity was assayed using intact erythrocytes as enzyme source. The cell : medium ratio during incubation was usually 1 : 100. The standard assay mixture contained 0.172 M Tris–HCl, 1% (w/v) defatted albumin and 1 mM emulsified [^{3}H]oleoylethanol (1.5×10^6 cpm) to 1 ml final vol. at 37°C (pH 7.6); the osmotic pressure was 295 ± 5 mOsm/kg. The release of [^{3}H]oleic acid during incubation was monitored as in [3]. Monoacylglycerol lipase activity was assayed with 1 mM mono-[^{3}H]oleoylglycerol as substrate in a reaction system identical to that used for [^{3}H]oleoylethanol except that an additional thin-layer chromatography was required for the purification of [^{3}H]oleic acid [4]. Diacylglycerol lipase activity was assayed with di-[^{3}H]oleoylglycerol as substrate, as in [5]. Enzyme kinetics were of zero order over the 10 min period of the assays. For each value of activity, duplicate assays performed with erythrocytes, erythrocyte ghosts or plasma as enzyme sources were reproducible within 10% of [^{3}H]oleic acid released. The lower limit of assay sensitivity was 0.01 munit of lipase activity. One munit of activity corresponds to the release of one nmole of acid per min. ^{3}H radioactivity was measured with a yield of 80%.

Attempts to solubilize the lipase were carried out by incubating the erythrocyte suspension (~10^8 cells/ml medium) for 30 min at 37°C with 1 mg/ml trypsin, 0.2 mg/ml chymotrypsin, 20 U/ml elastase and 0.5 mg/ml papaine; prior to use, papaine was activated by incubation for 15 min at 0°C with 5 mM cysteine and 0.03 mM dithiothreitol. Lipase activity was then assayed in the cell-free fluid. All proteolytic enzymes were from Sigma.

3. Results

Intact erythrocytes hydrolyzed [^{3}H]oleoylethanol at activity rate nearly constant during 20 min incubation (fig.1a). The optimum pH of the bulk phase was 7.5 ± 0.2. The dose–response curve was linear up to 3×10^8 cells added as enzyme source (fig.1b). The lipase was cell-bound during hydrolysis. This was shown by incubating the cells for 10 min at 37°C in medium identical to that used for the lipase assay, but containing no substrate [6]. After incubation, the suspension was centrifuged at $1500 \times g$ for 3 min. Lipase activity in the cell-free fluid was less than 5% of the total activity in the incubation mixture (cells+ fluid).

Lipase activity averaged 90 munits (range 76–120 munits for 6 experiments) per ml of packed cells (~1.6×10^{10} cells). Over 80% of this activity was recovered in the cell ghosts, whereas < 10% was found in the membrane-free hypotonic lysate, indicating

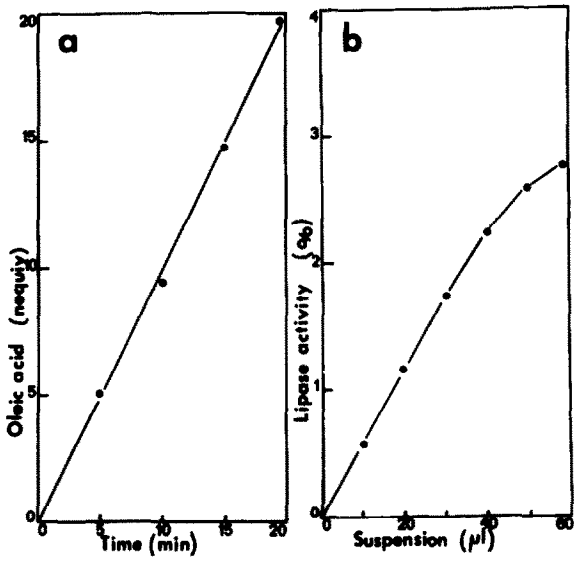

Fig.1. Lipolytic activity of intact rat erythrocytes toward an extracellular emulsion of [^{3}H]oleoylethanol: (a) Time course of hydrolysis with 20 µl cell suspension (~1.6×10^8 cells) added as enzyme source; (b) Hydrolysis curve as a function of the amount of enzyme. The erythrocyte suspension contained about 0.8×10^7 cells/µl. Fractions of the incubation mixture were taken 10 min after the addition of cells, and analyzed for their content of [^{3}H]oleic acid. No hydrolysis occurred during incubation of the substrate in the absence of cells.

that essentially all the lipase was in the membrane. The enzyme was neither released nor inactivated upon treatment with proteolytic enzymes (see section 2). Incubation of intact erythrocytes (1.6×10^8 cells/ml for 10 min at 37°C) with heparin (100 µg/ml) caused no release of the enzyme into the medium. No transfer of [^{3}H]oleic acid from the extracellular medium to the cellular lipids was detectable under the experimental conditions.

Comparative experiments showed that the cell-bound lipase was different from a monoester lipase present in rat plasma in the basal state. The ratio of oleoylethanol to monooleoylglycerol lipase activities averaged 0.4 for the cell-bound enzyme, versus nearly 3 for the plasma enzyme (table 1). Table 2 shows that while lipase activity increased in rat plasma from birth to adulthood, activity levels in erythrocytes did not vary. Table 3 shows that lipase levels in plasma were found to be high in rodents, low in other species and hardly detectable (if any) in human subjects; the profile for the cell-bound lipase is clearly different.

Since the presence of a diacylglycerol lipase has been reported in erythrocyte membranes [1], it was of interest to compare mono- and diester lipase activities. Intact erythrocytes hydrolyzed oleoylethanol at a rate ~4-times higher than dioleoylglycerol. Figure 2 shows that each activity elicited a different pattern of heat inactivation. After 15 min incubation at either 55°C or 60°C, diester lipase was inhibited by 40% and 80%, respectively, as compared to 10% and 40% for monoester lipase. Likewise, the two activities were differentially inhibited upon incubation of erythrocyte ghosts in the presence of 0.1% Triton X-100. After 15 min incubation at 37°C, < 20% of diester lipase had been preserved, versus 50% for monoester lipase. Taken together, the observed differences suggest that each activity is referable to a distinct catalytic protein.

4. Discussion

The results show that intact erythrocytes are able to catalyze the hydrolysis of a lipid emulsion added to the extracellular medium. The membrane-bound lipase active toward oleoylethanol is most probably the same enzyme which hydrolyzes monoacylglycerol

Table 1
Differential substrate specificity of erythrocyte-bound and plasma monoester lipases in adult rats

Substrate	Erythrocyte-bound lipase (munits/ml packed cells)	Plasma lipase (munits/ml plasma)
Oleoyl-ethanol	86	195
Monooleoyl-glycerol	200	62

Whole intact erythrocytes (~1.6×10^8 cells) or plasma (25 μl) were incubated for 10 min with the indicated substrates as in section 2. Two to four assays contributed to each value. Activity values agreed with 6% and 11% for the oleoyl-ethanol and monooleoylglycerol assays, respectively

Table 2
Erythrocyte-bound and plasma lipase activities against oleoyl-ethanol in rats as a function of age

Age (days)	Erythrocyte-bound lipase (munits/ml packed cells)	Plasma lipase (munits/ml plasma)
1	104	12
10	89	60
20	92	99
60	98	160

Incubations as in table 1. Each value is the mean of two experiments performed in duplicate. Values agreed within 10% and 14% for the erythrocyte-bound and plasma lipases, respectively

Table 3
Comparative erythrocyte-bound and plasma lipase activities against oleoyl-ethanol in various adult vertebrates

	Human	Rat	Rabbit	Chicken	Frog
Erythrocyte-bound lipase (munits/ml packed cells)	19	86	20	49	54
Plasma lipase (munits/ml plasma)	0.66	220	88	2.6	5.5

Appropriate aliquots of whole intact erythrocytes ($1.5–2.5 \times 10^8$ cells) or plasma (25–100 μl) were incubated for 10 min as in section 2. All lipolytic processes showed a broad maximum of activity between pH 7.3 and 8.0; they were run at pH 7.6. Two assays contributed to each value. Activity values agreed within 9% and 15% for the erythrocyte-bound and plasma lipase, respectively

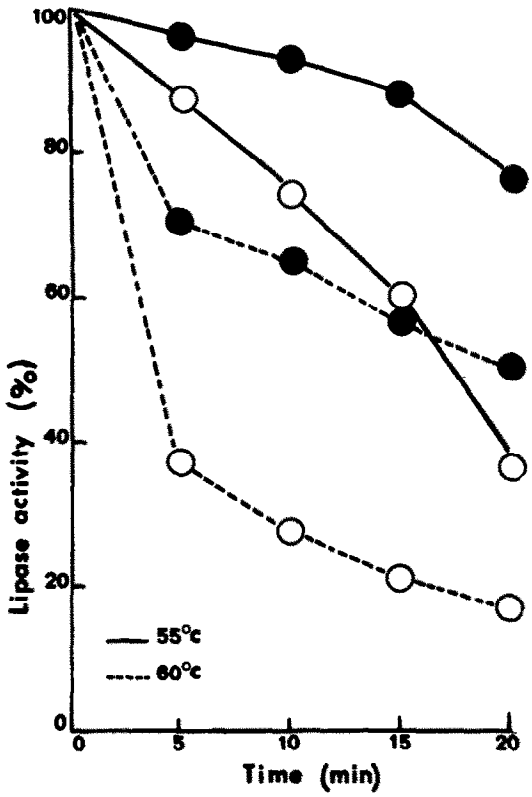

Fig.2. Variation of the measured monoester (•) and diester (○) lipase activities of ghost preparations (~ 15 μg protein) from rat erythrocytes upon incubation at 55°C and 60°C. Aliquots were pipetted from the treated preparations at the indicated times and immediately assayed at 37°C. Activities are expressed as % of control (zero-time) values measured in ghost preparations kept at 4°C, before incubation. Zero-time values were 41 and 9.1 munits/ml packed cells for mono- and diester lipase activities, respectively.

[7]. The choice of the former substrate, totally water-insoluble, ascertains that the monoester acylhydrolase is a true lipase [8]. The enzyme does not require the presence of serum for activity. No triacylglycerol lipase activity was detectable in rat erythrocytes under the experimental conditions. White blood cells and platelet suspensions exhibited lipase activities, but at much lower levels than erythrocytes.

The in vitro assay of membrane-bound lipases has been described so far with liver [6], fat [9] and heart [10] intact cells as enzyme sources. In these studies, the lipolytic process is likely to duplicate physiological conditions. Isolated fat cells, for example, hydrolyze extracellular lipids and incorporate the fatty acid chains [9]. The physiological role of membrane-bound lipases in erythrocytes is unknown. Very small amounts of neutral lipids are present in erythrocytes, and solely detectable in the plasma membrane [11]. As suggested [1], the bound lipases may act on these intramembranous lipids. Erythrocytes, however, contain monoester lipase activity at relatively high levels, comparable on a weight basis to those found in adipocytes (50–100 munits/g packed cells). It is worth considering the possibility that the erythrocyte lipase(s) plays a role in the hydrolysis of acylglycerol from the circulating lipoproteins.

Acknowledgements

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