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Acid phospholipase A activities in rat hepatocytes

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Cultured rat hepatocytes exhibit acid phospholipase A activity. On the basis of product formation from stereospecifically radiolabeled phosphatidylethanolamine substrates, phospholipases A_1 and A_2 have been identified with optimal activities at pH 4.5. According to subcellular fractionation studies, the acid phospholipases in hepatocytes appear to be located in the lysosomal compartment. Application of specific inhibitors of the biosynthesis, glycosylation, and translocation of lysosomal enzymes in hepatocyte cultures suggests a half-life of approx. 1 day for the acid lysosomal phospholipase A_1. About the same value for the half-life was obtained for the lysosomal marker enzymes, acid phosphatase and *β-N*-acetyl-D-hexosaminidase.

Hepatocyte; Lysosome; Marker enzyme; Phospholipase; Cycloheximide; Tunicamycin; Monensin

1. INTRODUCTION

Acid phospholipases A and C are unequivocal constituents of rat liver lysosomes [1–3]. The lysosomal phospholipase A_1 has also been purified to near homogeneity from this species [4,5]. However, liver is an organ composed of heterogeneous cell types [6,7]. It was therefore of interest to determine which cell types contain lysosomal acid phospholipases. Without presenting experimental proof, lysosomal acid phospholipase A_1 was reported to be present in hepatocytes as well as in Kupffer cells of rat liver [4]. Here, we provide evidence that acid phospholipases A_1 and A_2 are present in hepatocyte lysosomes, and that lysosomal phospholipase A_1 turnover in isolated rat hepatocytes seems to equal the turnover rates of the two lysosomal marker enzymes, acid phosphatase and acid *β-N*-acetyl-D-hexosaminidase.

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2. MATERIALS AND METHODS

2.1. *Substances*

All reagents and solvents used were of analytical grade and obtained from Sigma (München). [1-^{14}C]Palmitic acid (57 mCi/mmol) and [1-^{14}C]linoleic acid (56 mCi/mmol) were purchased from Amersham Buchler (Braunschweig). Syntheses of radiolabeled phospholipid substrates were carried out as described in [8]. 1-[1-^{14}C]Palmitoyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine (1-[^{14}C]PE) had a specific activity of 2617 dpm/nmol, that of 1-acyl-2-[(1-[^{14}C]linoleoyl-*sn*-glycero-3-phosphorylethanolamine (2-[^{14}C]PE) being 2214 dpm/nmol. Culture dishes were from Becton Dickinson (Heidelberg). Dulbecco's modified Eagle's medium was obtained from Gioco BRL (Eggenstein), Hepes from Biomol (Ilvesheim), silica gel H from Merck (Darmstadt), Triton X-100 from Serva (Heidelberg), and fetal calf serum from Biochrom (Berlin).

2.2. *Preparation and incubation of hepatocytes*

Hepatocytes were prepared by collagenase (type IV from *Clostridium histolyticum,* Sigma) perfusion of rat livers according to Seglen [9], washed, and suspended in Dulbecco's modified Eagle's medium to a final concentration of 10 mg/ml (w/v) which corresponded to about 10^6 cells/ml. The suspension medium was supplemented with $NaHCO_3$ (1.5 mg/ml), Hepes (3.6 mg/ml), penicillin (10^4 U/ml), streptomycin (0.1 mg/ml), dexamethasone (10^{-5} M), insulin (10^{-5} M) and 10% fetal calf serum, prior to use. Yields of cells averaged $7 \pm 4 \times 10^8$ and the suspension was 98–99% pure with regard to non-parenchymal cells. Cell viability was 96 ± 2% as assessed by the trypan-blue exclusion test. Aliquots of 3 ml of the cell suspension, each corresponding to approx. 3×10^6 hepatocytes, were

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placed in culture dishes (60 mm diameter). The cells were distributed evenly over the plate by gently swirling and rocking of the suspension, and then allowed to settle, forming a single continuous layer on the surface of the plate. The cells were kept at 37°C with continuous gassing (95% O_2, 5% CO_2) in a humidified atmosphere. After 3 h, non-attached cells were removed and fresh medium was added from which the fetal calf serum was omitted. Total duration of culture was up to 48 h.

2.3. *Experimental design*

Experiments were performed on the second day of cell culture. Media containing various inhibitors of protein biosynthesis, glycosylation, processing or transport, were adjusted with respect to osmolality (295 mosm/l) and pH (7.5). After incubation, the cell layer was washed three times with phosphate-buffered saline and the cells were scraped off with a rubber policeman in 1 ml of 0.25 M sucrose. Aliquots of this suspension were taken for determination of acid phospholipase activity, lysosomal marker enzyme activities and DNA content.

2.4. *Enzyme activities*

Phospholipase activity towards sonicated radioactive phosphatidylethanolamine at pH 4.5 was determined as in [10]. In a total volume of 250 μl the reaction mixture contained 20 mM sodium acetate buffer (pH 4.5), 10 μg protein (hepatocyte homogenate or subcellular fraction) and 200 μM sonicated radiolabeled phosphatidylethanolamine (1-[^{14}C]PE or 2-[^{14}C]PE). The time of reaction was adjusted to allow 5–10% hydrolysis in the samples, and was 120 min for hepatocyte homogenates. After incubation at 37°C, the substrate and the products of hydrolysis were extracted, separated from each other by thin-layer chromatography, and quantitatively determined by radioactivity measurements [10]. The lysosomal marker enzymes acid phosphatase (EC 3.1.3.2), acid β-*N*-acetyl-D-hexosaminidase (EC 3.2.1.52), and acid β-glucuronidase (EC 3.2.1.31) were determined according to Löffler et al. [11].

2.5. *Subcellular fractionation*

Isolated hepatocytes suspended in 0.25 M sucrose were disintegrated according to Löffler et al. [12] and fractionated as described by De Duve et al. [13].

2.6. *Analytical methods*

Protein was determined by the method of Lowry et al. [14] using bovine serum albumin as standard. DNA was measured as described by Hill and Whatley [15].

3. RESULTS AND DISCUSSION

When measured under standard assay conditions at pH 4.5 and towards phosphatidylethanolamine (50 μM), acid phospholipase activity in disintegrated rat hepatocytes amounted to 0.15 ± 0.04 mU/mg protein (18 experiments). This value is statistically not different from 0.18 ± 0.02 mU acid phospholipase activity/mg protein in total rat liver [3]. Analysis of the products formed

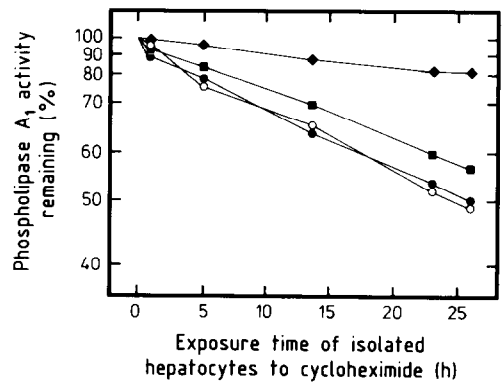

Fig.1. Effect of cycloheximide on the activities of acid phospholipase A_1 and lysosomal marker enzymes in cultured rat hepatocytes. Hepatocytes isolated by collagenase perfusion were cultured for 24 h as described in section 2 and subsequently incubated in the presence of cycloheximide (4×10^{-4} M). After various time intervals as indicated, the cells were washed and assayed for DNA content and enzyme activities. Data points (means of duplicates) represent percent of initial enzyme activity per unit DNA. (○) Acid phospholipase A_1, (●) acid phosphatase, (■) acid β-*N*-acetyl-D-hexosaminidase, (◆) acid β-glucuronidase.

from differently labeled radioactive phosphatidylethanolamine substrates indicated the presence of phospholipases A_1 and A_2. As calculated from the respective lysophospholipids that were liberated in those experiments, at least 68% of the parent phosphatidylethanolamines had been hydrolysed by an acid phospholipase A_1 and 25% by an acid phospholipase A_2. Since no stoichiometric ratios of the free fatty acids and the corresponding lysophospholipids were found, it is concluded that lysophospholipase(s) and/or phospholipase C were also active in hepatocytes at pH 4.5.

When isolated hepatocytes were disintegrated [12] and subjected to differential centrifugation according to De Duve et al. [13], each of the acid phospholipases as well as the lysosomal marker enzymes, acid phosphatase, acid β-*N*-acetyl-D-hexosaminidase and acid β-glucuronidase, was concentrated to about the same extent (4-fold) in the light mitochondrial L-fraction (not shown). This suggests a lysosomal localisation of acid phospholipase activity in hepatocytes, as was also expected from studies on total liver [1–3].

When isolated rat hepatocytes were cultured in the presence of specific inhibitors of the biosynthesis, glycosylation, or translocation of lysosomal

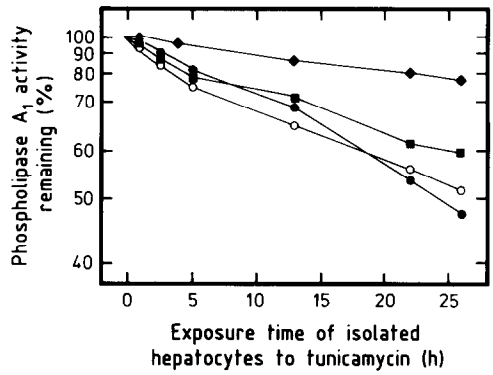

Fig.2. Effect of tunicamycin on the activities of acid phospholipase A_1 and lysosomal marker enzymes in cultured rat hepatocytes. Hepatocytes isolated by collagenase perfusion were cultured for 24 h as described in section 2 and subsequently incubated in the presence of tunicamycin (3 μg/ml). After various time intervals as indicated, the cells were washed and assayed for DNA content and enzyme activities. Data points (means of duplicates) represent percent of initial enzyme activity per unit DNA. (○), Acid phospholipase A_1, (●) acid phosphatase, (■) acid β-*N*-acetyl-D-hexosaminidase, (◆) acid β-glucuronidase.

enzymes, acid phospholipase A_1 activity (phospholipase A_2 activity was not determined in these experiments) decreased with time. This is shown, as an example, with the protein biosynthesis inhibitor cycloheximide (fig.1) and the glycosylation inhibitor tunicamycin (fig.2) which is known to lead to the secretion of newly synthesised unglycosylated lysosomal enzymes [16]. The time courses of the decrease in lysosomal enzyme activities in the presence of the monovalent cation ionophore monensin (not shown) were not different from those obtained with cycloheximide or tunicamycin. From the decrease in activity per unit of DNA, half-lifes of acid phospholipase A_1 and, for comparison, of the lysosomal marker enzymes, acid phosphatase, acid β-*N*-acetyl-D-hexosaminidase and acid β-glucuronidase, have been calculated. From experiments with either cycloheximide (4×10^{-4} M), tunicamycin (3 μg/ml), or monensin (10^{-5} M), mean half-lives were 26 ± 4 h for acid phospholipase A_1, 25 ± 4 h for acid phosphatase and 30 ± 2 h for β-*N*-acetyl-D-hexosaminidase. Thus, the turnover rates of these three lysosomal enzymes were not significantly different from each other, whereas that of β-glucuronidase significantly exceeded those of the other lysosomal enzymes (figs 1,2).

Taken together, our findings provide evidence of acid phospholipases A_1 and A_2 being lysosomal constituents of rat hepatocytes. They further suggest that the turnover kinetics of phospholipase A_1 closely parallel those of the two lysosomal marker enzymes, acid phosphatase and acid β-*N*-acetyl-D-hexosaminidase, whereas the turnover of acid β-glucuronidase appears to be much slower. The half-lives in hepatocytes of the lysosomal marker enzymes measured in the present studies have been calculated on the basis of enzyme activities per unit of DNA. They are in agreement with the half-lives calculated on the basis of pulse-labeling experiments (review [17].

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REFERENCES

[1] Van den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191–246.
[2] Van den Bosch, H. (1982) in: Phospholipids (Hawthorne, J.N. and Ansell, G.B. eds) pp. 313–357, Elsevier, Amsterdam, New York.
[3] Löffler, B.-M. and Kunze, H. (1987) FEBS Lett. 216, 51–56.
[4] Hostetler, K.Y., Yazaki, P.J. and Van den Bosch, H. (1982) J. Biol. Chem. 257, 13367–13373.
[5] Robinson, M. and Waite, M. (1983) J. Biol. Chem. 258, 14371–14378.
[6] Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell Biol. 72, 441–455.
[7] Van Berkel, T.J.C. (1979) Trends Biochem. Sci. 4, 204–205.
[8] Kunze, H., Nahas, N., Traynor, J.F. and Wurl, M. (1976) Biochim. Biophys. Acta 441, 93–102.
[9] Seglen, P.D. (1976) Methods Cell Biol. 13, 29–83.
[10] Kunze, H., Hesse, B. and Bohn, E. (1982) Biochim. Biophys. Acta 711, 10–18.
[11] Löffler, B.-M., Hesse, B. and Kunze, H. (1984) Anal. Biochem. 142, 312–316.
[12] Löffler, B.-M., Schmidt, M. and Kunze, H. (1987) Anal. Biochem. 163, 440–445.
[13] De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Apelmans, F. (1955) Biochem. J. 60, 604–617.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Hill, B.T. and Whatley, S. (1975) FEBS Lett. 56, 20–23.
[16] Rosenfeld, M.G., Kreibich, G., Popov, D., Kato, K. and Sabatini, D.D. (1982) J. Cell Biol. 93, 135–143.
[17] Skudlarek, M.D., Novak, E.K. and Swank, R.T. (1984) in: Lysosomes in Applied Biology and Pathology (Dingle, J.T. et al. eds) vol. 7, pp. 17–43, Elsevier, Amsterdam, New York.