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Phosphatidylinositol-Degrading Enzymes in Liver Lysosomes

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The major pathway by which liver lysosomal enzymes degrade phosphatidylinositol is through an EDTA-insensitive formation of phosphorylinositol. This is in distinct contrast with the Ca^{2+}-dependent production of phosphorylinositol from phosphatidylinositol, which is located in the cytosol. Lysosomal enzymes can also totally deacylate phosphatidylinositol, producing glycerophosphorylinositol.

The metabolism of inositol-containing phospholipids in animal tissue often differs from that of the major phospholipid fractions (Hawthorne, 1973). The rate of turnover of phosphatidylinositol is increased in a wide range of physiologically stimulated tissues (Hokin, 1968; Michell, 1975). Increases in phosphatidylinositol labelling by radioactive precursors are thought to arise indirectly as a result of cleavage of the phospholipid into diacylglycerol and phosphorylinositol [or cyclic phosphorylinositol; Dawson *et al.* (1971)]. This increase in degradation has been clearly documented in several tissues (Hokin, 1968; Hokin-Neaverson, 1974; Jones & Michell, 1974).

The enzyme thought to be responsible for this phosphatidylinositol hydrolysis is a phosphodiesterase, which is specific for phosphorylinositide and is dependent on Ca^{2+}. The enzyme is usually found in the cytosol (Dawson, 1959; Kemp *et al.*, 1961), but has also been reported in a plasma-membrane-enriched fraction in brain (Lapetina & Michell, 1973). We report in the present paper that the lysosomal fraction isolated from rat liver contains an enzyme that liberates phosphorylinositol from phosphatidylinositol and that can be clearly distinguished from the cytosolic enzyme. The lysosomal fraction also contains enzymes that deacylate phosphatidylinositol. These enzymes are equivalent to those described for the deacylation of nitrogen-containing phosphoglycerides (Franson *et al.*, 1971).

Methods

The subcellular fractionation of rat liver after the injection of Triton WR-1339 into the animal was substantially as described by Trouet (1974). ^{32}P-labelled phosphatidylinositol was prepared from *Saccharomyces cerevisiae* as described by Hazlewood & Dawson (1975). A portion of the [^{32}P]phosphatidylinositol solution (0.05 μmol, 10^5 d.p.m.) was taken to dryness at 40°C under vacuum, and to this was added 0.2ml of 0.1M-sodium acetate/acetic acid buffer, pH4.8, 0.05ml of 0.1M-EDTA and an appropriate portion of the enzyme fraction in a total volume of 0.8ml. At the end of the incubation (37°C for 30–60min) the test solution was cooled in ice, 0.05ml of 5% (w/v) serum albumin (bovine, fraction V; Armour Pharmaceutical Co., Eastbourne, U.K.) added and, after mixing, 0.5ml of 20% (w/v) trichloroacetic acid solution. After centrifuging (1000*g* for 5min), the supernatant was extracted four times with 2vol. of diethyl ether and then adjusted to pH7–8 with aq. NH_3. One-half of the solution was then ionophoresed as described by Dawson & Hemington (1977) in the presence of carriers of 1-phosphorylinositol and glycerolphosphorylinositol (10 μg of P) prepared as described by Dawson & Clarke (1972). The spots were located (Dawson & Clarke, 1972), cut out and the ^{32}P assayed directly in scintillation fluid (Unisolve; Koch–Light, Colnbrook, Bucks., U.K.).

Results

The main water-soluble radioactive product formed from [^{32}P]phosphatidylinositol by the lysosomal fraction was identified as phosphorylinositol. There were also lesser amounts of glycerophosphorylinositol and still smaller amounts of P_i. An enzyme that hydrolyses glycerophosphorylinositol into phosphorylinositol and glycerol has been described in mammalian tissue (Dawson & Hemington, 1977). However, the release of [^{32}P]phosphorylinositol from [^{32}P]phosphatidylinositol was unaffected by adding excess of unlabelled glycerophosphorylinositol at the start of the incubation. The release of phosphorylinositol by the lysosomal enzyme was not inhibited by EDTA, but was inhibited by Ca^{2+}, thus showing a clear distinction from the Ca^{2+}-dependent cytosolic enzyme liberating phosphorylinositol (Kemp *et al.*, 1961).

The distribution of EDTA-insensitive phospha-

Table 1. *Enzyme activities of rat subcellular fractions*

Subcellular fractions were assayed directly for cytochrome oxidase (Cooperstein & Lazarow, 1950), acid phosphatase (Trouet, 1974), glucose 6-phosphatase (Hübscher & West, 1965), 5′-nucleotidase (Michell & Hawthorne, 1965) and lactate dehydrogenase (Kornberg, 1955). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. These values are typical of results obtained in three independent experiments. Activities are expressed per mg of protein. n.d., Not detected.

Cell fraction	Cytochrome oxidase (μmol of cytochrome *c* oxidized/min per mg)	Acid phosphatase (μmol of P_i released/30min per mg)	Glucose 6-phosphatase (μmol of P_i released/20min per mg)	5′-Nucleotidase (μmol of P_i released/20min per mg)	Lactate dehydrogenase (μmol of NADH oxidized/min per mg)
Original homogenate	0.8	1.74	1.1	0.73	1.92
Nuclei	0.96	0.94	0.72	3.2	0.82
Mitochondria	3.49	0.56	0.43	0.07	0.06
Lysosomal fraction	0.27	58.6	0.43	1.0	0.04
Microsomal fraction	0.58	4.4	3.8	1.86	2.22
Supernatant	n.d.	0.42	0.02	0.17	2.75
Recovery of enzyme from original homogenate (%)	71	76	79	99	86

tidylinositol-degrading enzymes throughout the subcellular fractions of rat liver was investigated in a series of experiments. The animals had been previously injected with Triton WR-1339 to improve the separation and purity of the secondary lysosomes, although these were obtained in comparatively low yield. Table 1 lists some specific activities of marker enzymes for the six subcellular fractions isolated from the liver. It is apparent that the contamination of the lysosomal fraction by other cell fractions is very low. Only acid phosphatase (EC 3.1.3.2), the lysosomal marker, shows any appreciable enrichment over the original homogenate.

In preliminary experiments with these fractions, the EDTA-insensitive phosphatidylinositol degradation showed a much higher specific activity in the lysosomal fraction compared with the others. Thus in a typical experiment the following specific activities in μg of phosphatidylinositol P released as phosphorylinositol/h per mg of protein were obtained: nuclei, 0.003; mitochondria, 0.037; lysosomal fraction, 2.88; microsomal fraction, 0.05; supernatant, 0.017; total homogenate, 0.007). However, the enzyme activities in these fractions showed a 250–320% recovery compared with the original homogenate and we judged that large amounts of membrane in the latter might have interfered with the assay. To characterize the subcellular location of the enzyme more exactly, we therefore froze and thawed the six fractions listed in Table 1 five times, centrifuged them for 2h at 100000*g* and then dialysed them overnight. The resulting membrane-free supernatants were assayed for phosphatidylinositol-degrading activity, protein and marker enzymes. We assayed the fractions for phosphatidylinositol degradation in the presence of 5mM-Ca^{2+} at pH5.5 [conditions which are optimum for the phosphodiesterase described by Kemp *et al.* (1961)], or in the presence of 5mM-EDTA and at pH4.8 (optimum for the activity in lysosomes). For comparison, the enzyme markers assayed were acid phosphatase and lactate-dehydrogenase (EC 1.1.1.27).

The results shown in Fig. 1 clearly demonstrate the cytosolic distribution of the Ca^{2+}-dependent enzyme that liberates phosphorylinositol and the lysosomal distribution of the Ca^{2+}-independent enzymes that liberate both glycerophosphorylinositol and phosphorylinositol.

A further test that the EDTA-insensitive phosphatidylinositol degradation is located in the lysosomal fraction was to demonstrate latency of the activity (Silink & Rowe, 1975). Portions of a post-nuclear supernatant of liver were frozen and thawed up to eight times and then centrifuged for 2h at 100000*g*. The resulting supernatants were assayed for acid phosphatase and for the release of phosphorylinositol and glycerophosphorylinositol from phosphatidylinositol in the presence of 5mM-EDTA at pH4.8. The three curves relating enzyme activity and the number of freeze–thaw cycles (Fig. 2) could be fitted by curves of common slope without significant increase in variance.

Preliminary studies to test the specificity of the lysosomal enzyme have shown that, under the incubation conditions described above, phosphorylinositol is released from phosphatidylinositol at 15–20 times the rate of release of phosphorylcholine from phosphatidylcholine. The rate of formation of phosphorylethanolamine from phosphatidylethanolamine is even slower.

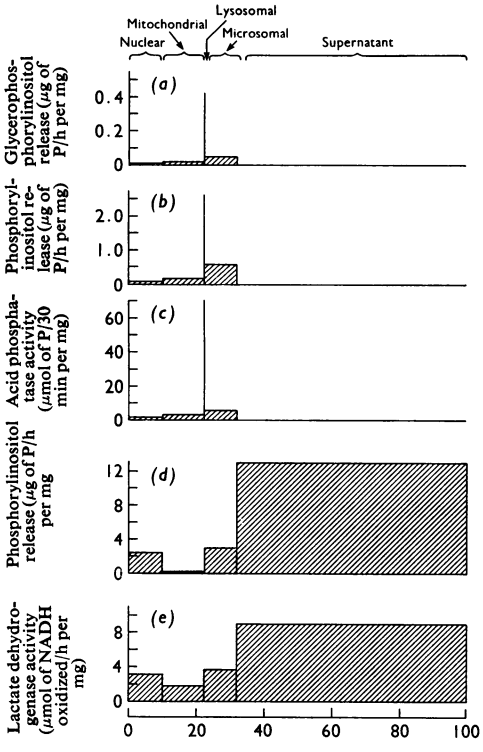

Fig. 1. *Distribution of phosphatidylinositol-degrading enzymes and marker enzymes in soluble proteins obtained from liver subcellular fractions*

(*a*) Glycerophosphorylinositol release in the presence of EDTA; (*b*) phosphorylinositol release in the presence of EDTA; (*c*) acid phosphatase activity; (*d*) phosphorylinositol release in the presence of 5mM-Ca^{2+}; (*e*) lactate dehydrogenase. The specific activities in the soluble fraction from the original homogenate, which contained 37mg of protein/g of liver, were as follows: (*a*) 0.056μg of P/h per mg of protein; (*b*) 0.18μg of P/h per mg of protein; (*c*) 1.61μmol of P/30min per mg of protein; (*d*) 5.8μg of P/h per mg of protein; (*e*) 6.15μmol of NADH/h per mg of protein. Similar results to these were obtained in an additional experiment.

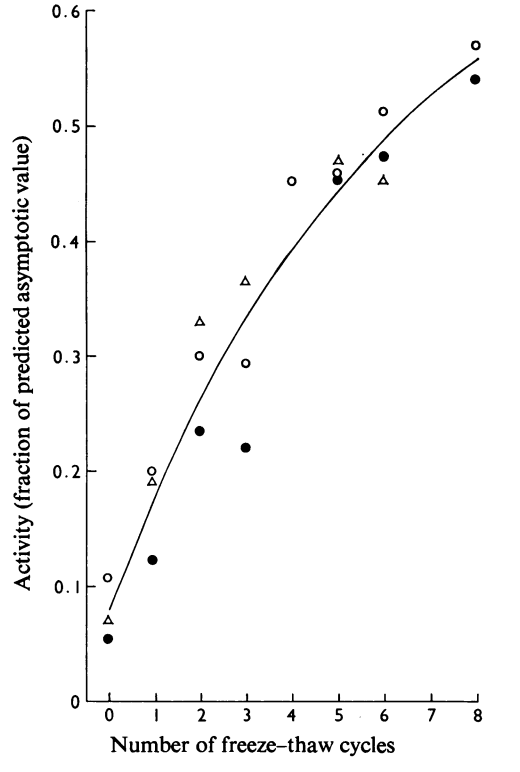

Fig. 2. *Relationship between enzymic activity liberated and number of freeze–thaw cycles*

○, Acid phosphatase. Phosphorylinositol formed (●) and glycerophosphorylinositol formed (△) by EDTA-insensitive phosphatidylinositol degradation. The enzyme preparation used was a liver post-nuclear supernatant. After freezing and thawing for the number of cycles indicated, the preparations were centrifuged (100000*g* for 2h) and the supernatants dialysed overnight. The activities (*y*) were scaled to a fraction of the asymptotic value predicted for a common slope ($r = 0.9082$) by using a standard exponential function $y = 0.7226 - 0.6466 \times (0.8349)^x$ to simplify graphical presentation.

Discussion

Fowler & de Duve (1969) showed that liver lysosomes could hydrolyse phosphatidylinositol into water-soluble products, but did not investigate the nature of these products. By inference from other phospholipids, it might be assumed that the reaction was a complete deacylation to form glycerophosphorylinositol; such lysosomal deacylating phospholipases have been investigated in some detail by using nitrogen-containing phosphoglycerides as substrates (Franson *et al.*, 1971). The present results clearly show that there is an enzyme system in liver which can completely deacylate phosphatidylinositol and which is lysosomally located. In addition, examination of the solvent-soluble portion of incubation mixtures shown an accumulation of lysophosphatidylinositol, which is the presumed intermediary in the pathway.

However, the dominant pathway of degradation of phosphatidylinositol by lysosomal enzymes is by the schism of phosphorylinositol from the substrate. Presumably diacylglycerol is the other product, although we have not yet been able to demonstrate

an accumulation of this neutral lipid, possibly because of its further hydrolysis. We have shown in subsidiary experiments that diacylglycerol can be broken down by liver lysosomal preparations, presumably by lipases of the type described by Hayase & Tappel (1970). These lipases are markedly stimulated by the presence of phosphatidylinositol (Karuja & Kaplan, 1973). The lysosomal location of the phosphorylinositol-producing enzyme can be deduced by its close correlation with acid phosphatase in the subcellular fractionation, its quantitatively similar latency to acid phosphatase on repeated freezing and thawing, and its pH optimum (4.8). It can be distinguished from the other enzyme producing phosphorylinositol in the presence of Ca^{2+} by the latter's correspondence with lactate dehydrogenase in the subcellular distribution studies (cytosolic; Fig. 1), its differing pH optimum (5.6; Kemp *et al.*, 1961) and its total inhibition by EDTA.

The physiological role of this enzyme in lysosomes forming phosphorylinositol can only be speculative at present. An increase in phosphatidylinositol breakdown by physiological stimuli is most clearly demonstrated in tissues when extracellular secretion by vesicles or storage granules is stimulated (Hokin, 1968; Hawthorne, 1973; Michell, 1975). The role of lysosomes in secretion, and the influence of the structure of their membranes on their function, have been studied in many biological systems (Dingle & Fell, 1969).

It is now increasingly apparent that the response of phosphatidylinositol to excitatory agents is sometimes independent of a rise in intracellular concentration of Ca^{2+} (Trifaró, 1969; Jones & Michell, 1975; Oron *et al.*, 1975; Jafferji & Michell, 1976). The activity of the present enzyme must clearly therefore be a candidate for the increased breakdown of phosphatidylinositol observed in many states of cellular hyperactivity.

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