ENDOGENOUS ACYLHYDROLASE BREAKS DOWN DIGLYCERIDE IN 5° PHOSPHOLIPASE C TREATED RAT LIVER MICROSOMES. PRODUCTS INHIBIT GLUCOSE 6-PHOSPHOHYDROLASE

B.R. CATER and T. HALLINAN

Department of Biochemistry, Royal Free Hospital School of Medicine, University of London, Hunter Street, London WC1N 1BP, England

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1. Introduction

A biphasic inhibition of glucose 6-phosphohydrolase (G-6-Pase) occurs when rat liver microsomes are treated at 5° with *Cl. welchii* phospholipase C and then post-incubated without substrate in the presence of EGTA at either 20° or 37°. The 5°loss of G-6-Pase is in the range of 40–60% of the total activity and is completely restorable by the addition of sonicated lecithin. The second phase of inhibition is temperature dependent and results in the complete loss of the remaining G-6-Pase activity. It is not reversible by the addition of lecithin although adding lecithin prior to heating at 20° or 37° protects completely against phase II inhibition [1, 2]. Further investigations have now revealed an explanation of this heat labilization phenomenon.

While studying the effects of exogenous fatty acids and lysolecithin on G-6-Pase in untreated microsomes, it became evident that fatty acids cause a temperature dependent inhibition closely mimicking that described above. However, the concentrations required seemed too high to be generated by endogenous phospholipase A under the conditions of phospholipase C treatment (pH 6.8). Further investigations have now shown a twenty-four-fold increase in fatty acid content is produced by the breakdown of diglyceride, an end product of phospholipase C action previously supposed to be stable. It has also been established that this breakdown is due to endogenous microsomal acylhydrolase activity and that such a phenomenon is also evident on phospholipase C treatment of freshly collected rat erythrocytes. These results provide a probable explanation for the heat labilization phenomenon observed on phospholipase C treatment of microsomal G-6-Pase and further demonstrate the complexities surrounding the question of phospholipid dependence of this enzyme.

2. Experimental

All experiments were carried out on 150–250 g male rats. Preparation and radioactive labelling of microsomes was as previously reported [1]. Lipid extraction was by the method of Folch et al. [3]. Total lipid was used for TLC separation but the phospholipid, which interferes with the fatty acid assay as described by Duncombe [4], was removed with silicic acid [5]. Removal was at least 97% complete. Protein was assayed by the method of Lowry et al. [6].

3. Results

Either palmitate, oleate, linoleate or linolenate when present in a concentration of 0.1–0.4 μmoles/mg protein caused complete or partial inactivation of G-6-Pase on incubation at 37° in the absence of G-6-P (table 1). Linoleate appeared the most effective, exhibiting marked inhibitory effects when present in concentrations as low as 0.1 μmoles/mg protein, with linolenate being the next most effective and palmitate the least effective. Time course studies indicated that incubation in the presence of approx. 0.2 μmoles of

Table 1
Percentage inhibition of G-6-Pase by the addition of fatty acid.

Fatty acid concn. μmoles/mg microsomal protein	% Inhibition of G-6-Pase			
	Palmitate	Oleate	Linoleate	Linolenate
0	7.4	7.4	7.4	7.4
0.1	8.3	5.4	27.1	13.1
0.2	–	–	94.5	64.8
0.4	46.2	99.2	99.2	98.4

Fatty acids (Sigma) were suspended essentially by the method of Feihn and Hasselbach [9] although pH adjustment prior to and after sonication was found to be a useful modification. All sonications were carried out in ice under a nitrogen atmosphere using an MSE Ultrasonic Disintegrator. All incubations were at 37° for 60 min and contained 3.3 mM EGTA and 20 mM Tris-maleate buffer pH 6.8. G-6-Pase activity of the incubated microsomes is expressed as a percentage of the zero-time activity of controls without added fatty acid. Zero time inhibition was evident only with 0.4 μmoles of linoleate per mg microsomal protein. Linoleate and linolenate were assayed at 0.05 μmoles/mg microsomal protein but showed no inhibition.

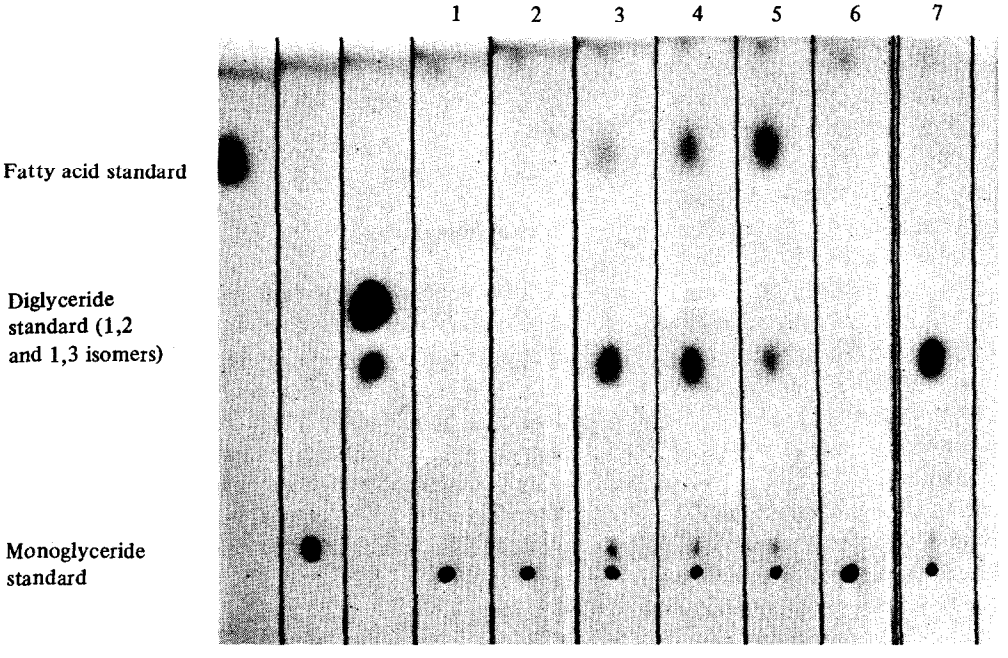

Fig. 1. TLC of lipid extracts: Adsorbent was silica gel H (Merck) activated for 1 hr at 110° immediately before use. Development was in petroleum ether: diethyl ether: acetic acid (70:20:4) after which the plate was dried and charred (see legend table 2).

linoleate per mg protein resulted in 60% inhibition after 15 min and total inhibition after 30 min. BSA was shown to be capable of partial protection when added prior to incubation but totally incapable of reversing inhibition if added after incubation. This too was the case if BSA was added prior to and after 37° post-incubation of 5° phospholipase C treated microsomes (Cater and Hallinan, unpublished).

The above evidence indicated a role for fatty acids in the heat labilization of phospholipid depleted G-6-Pase. However, the generation of sufficient concentration of fatty acid in the system, even if this were more effective when present endogenously, remained a problem. Further investigation has

Table 2
Fatty acid concentration of lipid extracts.

nmoles fatty acid/mg microsomal protein						
1	2	3	4	5	6	7
12	20	64	146	286	14	10

Table 2, fig. 1: Fatty acid accumulation on phospholipase C treatment of rat liver microsomes: Phospholipase treatment was carried out at 5° with 80–120 μg phospholipase C (*Cl. welchii;* Sigma) per mg microsomal protein. All incubations contained 1 mM $CaCl_2$ and 20 mM Tris-maleate buffer, pH 6.8. Phospholipase C action was terminated by making incubations 3.3 mM with respect to EGTA. (1) Control: containing phospholipase C, but with EGTA added at time zero. Zero time sample. (2) Control: containing phospholipase C, but with EGTA added at time zero. Incubated 60 min at 5° followed by 60 min at 37°. (3) Phospholipase C treated: 60 min at 5° prior to addition of EGTA. (4) Phospholipase C treated: 60 min at 5° prior to addition of EGTA followed by 10 min post-incubation at 37°. (5) Phospholipase C treated: 60 min at 5° prior to addition of EGTA followed by 60 min post-incubation at 37°. (6) Phospholipase C replaced by equivalent volume of distilled water. 60 min incubation at 5° prior to addition of EGTA followed by 60 min post-incubation at 37°. (7) Microsomes boiled prior to incubation; then incubated with phospholipase C at 5° for 60 min prior to addition of EGTA followed by 60 min post-incubation at 37°.

shown conclusively formation of fatty acid does occur at the expense of the diglyceride formed during the initial 5° phospholipase C action on the microsomes (table 2, fig. 1). Furthermore, this breakdown has been shown to be due to endogenous microsomal acylhydrolase action as it is absent when microsomes are boiled prior to phospholipase C treatment (table 2, fig. 1). In this case the diglyceride is formed normally but no further breakdown to fatty acids is evident. This result appears to effectively rule out the possibility that the diglyceride breakdown demonstrated in the system containing unboiled microsomes is due to a contaminant in the crude phospholipase C preparation used in these experiments. The contribution of endogenous phospholipase A action was assayed by replacing phospholipase C by water in the normal system. No significant fatty acid production occurred, thus indicating a negligible endogenous phospholipase A contribution. This is presumably because the pH of treatment with phospholipase C was deliberately chosen to be 2–3 pH units lower than the pH optimum for the microsomal phospholipase A [7, 8]. Acylhydrolase activity occurs at both 5° and 37° and does not require calcium ions. This is evidenced by a five-fold-increase in fatty acid concentration following phospholipase C treatment at 5° and an overall twenty-four-fold increase when they are further post-incubated at 37° for 60 min, the latter treatment being carried out in the presence of EGTA (table 2; fig. 1).

Preliminary studies indicate that diglyceride breakdown is not unique to liver microsomes as it was also demonstrable upon treatment of fresh rat erythrocytes with phospholipase C at 5° followed by post-incubation at 37°.

4. Discussion

It appears that the diglyceride which is generated by the treatment of rat liver microsomes with phospholipase C is further broken down by an endogenous acylhydrolase. Since phospholipase C is generally chosen as an investigative tool because none of its products are themselves significantly "membrane active", this finding, if generalized, might necessitate a substantial reappraisal of data already gained from the use of this enzyme. It certainly provides a self-consistent explanation of the heat labilization of G-6-Pase after phospholipid depletion by phospholipase C treatment. In addition, the ability of BSA to provide partial protection from initial 5° decline in activity of G-6-Pase on phospholipase C treatment may well indicate a fatty acid role in this phase of G-6-Pase inhibition as well (Cater and Hallinan, unpublished). If this proves to be the case it would seem no longer necessary to posit phospholipid dependence for G-6-Pase. Further investigations are now in progress to clarify this matter.

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