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HYDROLYSIS OF 1,2-DIGLYCERIDE BY MEMBRANE-ASSOCIATED LIPASE ACTIVITY DURING PHOSPHOLIPASE C TREATMENT OF MEMBRANES

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SUMMARY

1. When rat erythrocyte membranes were treated with high concentrations of phospholipase C, approximately 70% of the phospholipids were hydrolysed.

2. The main product of phospholipase C action, 1,2-diglyceride, did not accumulate even when 50% of the membrane phospholipid was hydrolysed in about 10 min.

3. During phospholipase treatment unesterified fatty acids and a variable amount of monoglyceride accumulated. Between 1.2 and 1.8 ester bonds were cleaved per molecule of phospholipid hydrolysed by the phospholipase C, suggesting that the membranes contained a highly active lipase capable of attacking 1,2-diglyceride and monoglyceride.

4. Hydrolysis of emulsified rat erythrocyte lipids with phospholipase C yielded 1,2-diglyceride but there was no hydrolysis of acyl ester bonds or liberation of unesterified fatty acids. Thus the lipase was not a contaminant in the phospholipase C preparations.

5. Rat erythrocyte membranes treated with phospholipase C decreased in size, developed lipid droplets on their surface and remained morphologically intact.

6. Hydrolysis of diglyceride generated in membranes by phospholipase C action was observed (in order of decreasing activity) in rat erythrocyte membranes, in microsomes, plasma membranes and other subcellular fractions from rat liver and in pig erythrocyte membranes, human erythrocyte membranes, rat muscle microsomes and myelin. In the latter three preparations only a small fraction of the diglyceride was hydrolysed.

7. These results are discussed in relation to the extensive use of phospholipase C in studies of the structure of biological membranes.

INTRODUCTION

Phospholipase C has been widely used in studies of membrane structure and function as a tool for modifying membrane lipids (for examples refs 1–6). Its action causes the conversion of the relatively polar glycerophospholipids and sphingomyelin of the membranes to 1,2-diglyceride and ceramide, respectively. These products

seem unlikely to damage the membranes, as they are relatively non-polar and they appear to separate rapidly from the main lipoprotein structure of the membranes as discrete lipid droplets. Interpretation of information from such experiments has until now been mainly derived from membranes in which these products were stable and in which consideration of effects related to further breakdown of the diglyceride or ceramide was unnecessary. However, we have now observed that in rat erythrocyte membranes and in some other membranes hydrolysis of 1,2-diglyceride can occur, leading to an accumulation of unesterified fatty acids in the preparations; such effects need to be considered in studies of membranes using this phospholipase.

A preliminary account of some of these experiments has been published[7].

METHODS

Preparation of erythrocyte membranes

Rat and pig blood were obtained fresh, using heparin or acid citrate–dextrose as anticoagulant, and human blood in acid citrate–dextrose was obtained from a local blood bank within a week of its collection. The blood was centrifuged and the cells washed 2–4 times with 4 vol. of 0.9% NaCl, the upper layer of the pellet being discarded each time. The cells were suspended in an equal volume of 0.9% NaCl and were then lysed by the addition to 1 vol. of cell suspension of 10 vol. of ice-cold 10 mM Tris–HCl, pH 7.4. After standing for 5 min the membranes were sedimented at $8200 \times g$ for 10 min, and were washed 2–4 times with 10 vol. 25 mM Tris–HCl, pH 7.4, centrifuging each time for 10 min at $8200 \times g$. The final pellet was suspended in the 25 mM buffer to a volume of 1–2 ml per ml cells lysed.

Rat and human erythrocyte membranes were also prepared in bicarbonate buffer (80 ideal mosM, pH 7.4)[8].

Preparation of other membranes

Primary cell fractions from rat liver were isolated as described previously[9], as were rat liver plasma membranes[10] and rat muscle microsomes[11]. The rat cerebral cortical myelin preparation was fraction M_1 0.8 of Rodriguez de Lores Arnaiz *et al.*[12].

Treatment of samples with phospholipase C

Clostridium welchii phospholipase C (obtained from Sigma Chemical Co.) was used, usually at a concentration of 100 μg/ml, in 25 mM Tris–HCl, pH 7.4, containing 0.75 mM $CaCl_2$. The membrane suspension or lipid emulsion was incubated under these conditions for 60 min at 37 °C (unless otherwise specified) together with a control sample from which the phospholipase C was omitted. Samples for microscopy were incubated at room temperature (approximately 21 °C) to avoid the clumping which occured at higher temperatures. Membrane lipids from samples treated with phospholipase C at 21 °C were similar in composition to those from membranes treated at 37 °C.

Extraction and analysis of lipids

Lipids were extracted as described previously[10] and dissolved in chloroform. Thin-layer chromatography of the non-polar lipids and phospholipids was by the methods of Skipski and co-workers[13,14] phosphate analysis by the methods of King[15] and of Galliard *et al.*[16] and acyl ester assay by that of Snyder and Stephens[17].

RESULTS

The composition of the lipids of membranes treated with phospholipase C

A range of different membrane preparations was treated with phospholipase C and the lipids were extracted and examined by thin-layer chromatography (Table I and Fig. 1). In most of the membranes the bulk of the newly produced lipid was, as expected, 1,2-diglyceride and there was little unesterified fatty acid present. There were, however, two exceptions. These were rat liver microsomes, in which approximately equal amounts of unesterified fatty acid and 1,2-diglyceride were present and rat erythrocyte membranes, where there were large quantities of unesterified fatty acids and little or no visible diglyceride. The rat erythrocyte membranes were examined further to determine the properties of the system which was releasing these fatty acids.

Investigations of the labelled products after phospholipase C treatment of [^3H]palmitate-labelled rat erythrocyte membranes

Washed rat erythrocytes were incubated with [^{3}H]palmitic acid under conditions similar to those used by Shohet *et al.*[18] to label the phospholipids (mainly the phosphatidylcholine). Membranes were isolated from the labelled cells and treated with phospholipase C. There was a rapid decrease in the amount of labelled

TABLE I

THE LIPIDS ACCUMULATED DURING PHOSPHOLIPASE C-TREATMENT OF VARIOUS MEMBRANES

Membranes were isolated in the medium indicated (see Methods). All membranes were treated with phospholipase C under similar conditions (100 μg/ml phospholipase C; 0.75 mM $CaCl_2$; 25 mM Tris–HCl, pH 7.4; 60 min, 37 °C). The approximate amount of each component was assessed visually from the relative staining intensities of separated lipids in samples separated by thin-layer chromatography[13] and then exposed to iodine vapour.

Type of membrane	*Source*	*Isolation medium (anticoagulant)*	*1,2-Diglyceride*	*Ceramide + Monoglyceride*	*Unesterified fatty acid*
Erythrocyte	Rat	Tris–HCl or HCO_3^- (Heparin or acid citrate–dextrose)	±	++	+++++
	Pig	Tris–HCl (acid citrate–dextrose)	+++	+	++
	Human	Tris–HCl or HCO_3^- (acid citrate–dextrose)	++++	++	+
Liver microsomes (and other primary fractions)	Rat	Sucrose	+++	+	+++
Liver plasma membranes	Rat	Sucrose	+++	+	+++
Skeletal muscle microsomes	Rat	KCl	+++++	±	±
Cerebral cortical myelin	Rat	Sucrose	+++++	++	±

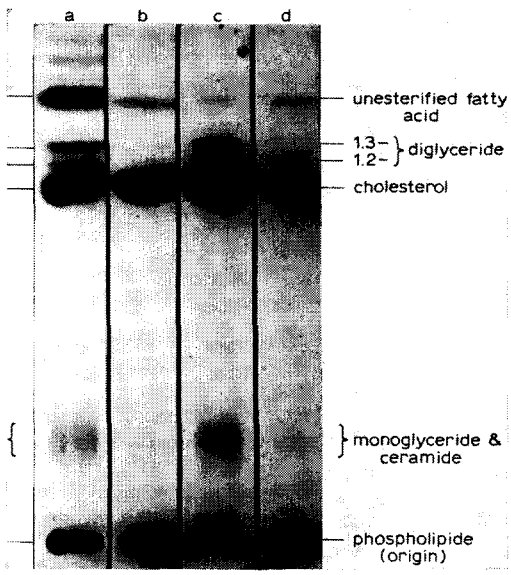

Fig. 1. Rat erythrocyte membranes (a,b) and lipids extracted from such membranes (c,d) were incubated with phospholipase C (a,c) or without phospholipase C (b,d). Lipids were extracted and separated by thin-layer chromatography[13]. The mobilities of standards of phospholipid, ceramide, monoglyceride, cholesterol, 1,2-diglyceride, 1,3-diglyceride, and unesterified fatty acids are indicated.

phospholipids, which correlated quite well with the decrease in lipid phosphorus (Fig. 2a). Simultaneously there was an accumulation of two ^{3}H-labelled lipids which appeared to be an unesterified fatty acid and either a monoglyceride or ceramide. This increase was approximately equal to the decrease in radioactivity of the phospholipids. No accumulation of radioactivity was observed in the 1,2-diglyceride fraction (Fig. 2b).

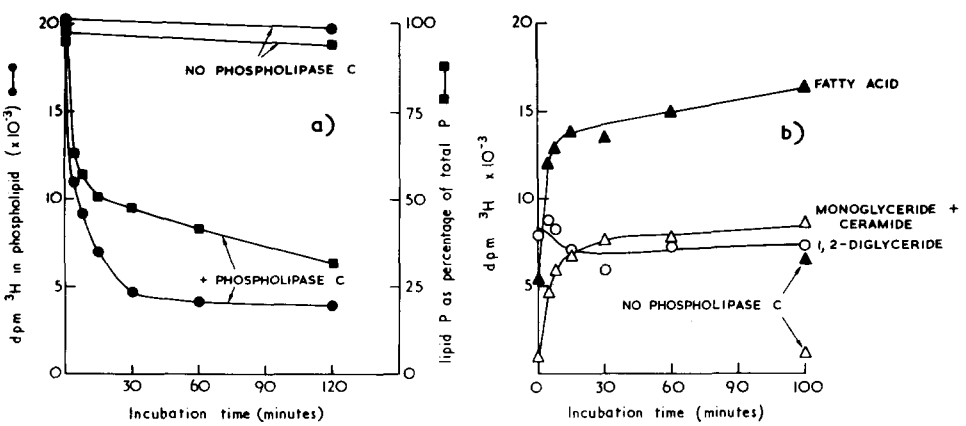

Fig. 2. [^{3}H]palmitate-labelled rat erythrocyte ghosts (see text) were treated with phospholipase C. The decrease in phospholipid content (■—■) and phospholipid radioactivity (●—●), was accompanied by a rise in the radioactivity of fatty acid (▲—▲) and of monoglyceride + ceramide (△—△), but not of 1,2-diglyceride (○—○).

Treatment with phospholipase C of lipids extracted from rat erythrocyte membranes

The lipids were extracted from a sample of membranes, emulsified and then treated with phospholipase C under the same conditions as were used with the membranes; the main product of the reaction appeared to be 1,2-diglyceride. There was no marked accumulation of unesterified fatty acid (Fig. 1).

In a similar experiment in which lipids extracted from [^{3}H]palmitate-labelled membranes were used, there was clearly a transfer of radioactivity from the phospholipids to 1,2-diglyceride, but no additional label appeared in unesterified fatty acids (Table II).

TABLE II

PHOSPHOLIPASE C TREATMENT OF LIPIDS EXTRACTED FROM [^{3}H]PALMITATE-LABELLED RAT ERYTHROCYTES

Erythrocytes were obtained and incubated for 2 h in Krebs–Ringer bicarbonate containing ^{3}H-labelled palmitate complexed to bovine serum albumin essentially as described by Shohet *et al.*[18]. Cell membranes were isolated in 25 mM Tris–HCl, pH 7.4. The lipids were extracted and emulsified in 25 mM Tris–HCl, pH 7.4, and the emulsion treated with phospholipase C as described under Methods. The lipids were isolated by thin-layer chromatography and their radioactivities determined.

	Disintegrations ^3H/min		
	Before phospholipase treatment	*After phospholipase treatment*	*Change*
Phospholipids	15100	3560	−11540
1,2-Diglyceride	8200	22400	+14200
Monoglyceride + ceramide	105	143	+38
Unesterified fatty acids	880	930	+50

Changes in carboxylic ester and phosphate content of the lipids of rat and human erythrocyte membranes during phospholipase C treatment

When rat erythrocyte membranes were treated with phospholipase C there was a marked decline in their content of ester bonds (Table III). This confirmed that the diglyceride produced during phospholipase treatment was being further degraded

TABLE III

DECREASE IN THE PHOSPHORUS AND ACYL ESTER CONTENT OF RAT ERYTHROCYTE MEMBRANE LIPIDS DURING TREATMENT WITH PHOSPHOLIPASE C

Membranes prepared in 25 mM Tris–HCl, pH 7.4, were incubated with phospholipase C at 37 °C as described under Methods and their lipids analysed. Values are average (with the range of values observed) from 7 experiments.

	Percentage decreases (range of values)
Lipid phosphorus	71 (57–81)
Lipid acyl ester	52 (37–65)

to unesterified fatty acid and monoglyceride, and subsequently to glycerol. This hydrolysis was somewhat variable in different preparations, but was always substantial. In human or pig erythrocyte membranes there was a decrease in the ester content of the lipids, but this was much smaller than in rat membranes. Clearly most of the diglyceride produced by phospholipase C in these systems was not being hydrolysed further.

The morphology of phospholipase C-treated rat erythrocyte membranes

The rat erythrocyte membranes were examined by phase-contrast microscopy. They were a fairly uniform population of spherical profiles, which showed no tendency to fragment. During treatment with phospholipase C the membranes remained spherical, but decreased considerably in diameter. Simultaneously refractile droplets appeared on the membranes.

DISCUSSION

The products of the action of phospholipase C on the glycerophospholipids of membranes are 1,2-diglycerides and water-soluble phosphate esters. The detection of significant quantities of unesterified fatty acids and of monoglycerides in the lipids extracted from membranes treated with phospholipase C therefore indicates the additional presence of a diglyceride lipase in these systems. Two experimental observations clearly show that this lipase activity is a property of the membranes, rather than a contaminant in the phospholipase C. First, the level of activity varied widely with different membrane preparations. Secondly, unesterified fatty acids were not detected when lipids were first extracted from the membranes, then emulsified in water and treated with phospholipase C. The presence in rat liver plasma membranes of enzymes capable of hydrolysing glycerides has previously been reported[19,20], but such activity had not been noted in erythrocyte membranes. Cater and Hallinan[21,22] have observed, in experiments similar to ours, the production of unesterified fatty acids in phospholipase C-treated liver microsomes and have pointed out the importance of this observation in relation to the modification of some membrane-localised enzyme activities. It seems likely that in all the membranes examined thus far the levels of diglyceride lipase activity could produce enough fatty acids and monoglycerides to significantly modify the activities of some membrane-bound enzymes (see, for example Cater *et al.*[23]).

Only in rat erythrocyte membranes and in rat liver cell fractions was the level of diglyceride lipase activity sufficient to degrade the major part of the diglyceride produced by the action of phospholipase C on the membrane preparations. Thus the previous identification of the dense droplets associated with phospholipase C-treated human erythrocyte membranes[6] and muscle microsomes[2] as consisting of 1,2-diglyceride is justified. However, rat erythrocyte membranes treated with phospholipase C also show droplet formation (see Results and ref. 6), and these droplets clearly cannot consist of diglyceride. Such droplets could contain ceramide (derived from sphingomyelin) and monoglyceride, and a similarity has been noted between the fine structure of these droplets as seen in sections by electron microscopy and that of ceramide trihexoside droplets present in tissues of patients with Fabry's disease[6]. Recent observations by Meldolesi *et al.*[24,25] on the morphology of lipase-

digested hepatic and pancreatic endoplasmic reticulum fragments have shown that these membranes also have associated dense droplets, the appearance of which was prevented by the presence of bovine serum albumin. Under such conditions extensive release of unesterified fatty acids from lipids occurred, but these fatty acids were complexed by serum albumin. It therefore seems likely that both in their studies and in the present experiments, major constituents of the droplets were unesterified fatty acids.

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REFERENCES

1 Lenard, J. and Singer, S. J. (1968) *Science*, 159, 738–739
2 Finean, J. B. and Martonosi, A. (1965) *Biochim. Biophys. Acta* 98, 547–553
3 Emmelot, P. and Bos, C. J. (1968) *Biochim. Biophys. Acta* 150, 341–353
4 Ottolenghi, A. C. and Bowman, M. H. (1970) *J. Membrane Biol.* 2, 180–191
5 Duttera, S. M., Byrne, W. L. and Ganoza, M. C. (1968) *J. Biol. Chem.* 243, 2216–2228
6 Coleman, R., Finean, J. B., Knutton, S. and Limbrick, A. R. (1970) *Biochim. Biophys. Acta* 219, 81–92
7 Michell, R. H. and Coleman, R. (1971) *Biochem. J.* 124, 49 P
8 Bramley, T. A., Coleman, R. and Finean, J. B. (1971) *Biochim. Biophys. Acta* 241, 752–769
9 Michell, R. H. and Hawthorne, J. N. (1965) *Biochem. Biophys. Res. Commun.* 21, 333–338
10 Coleman, R., Michell, R. H., Finean, J. B. and Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* 135, 573–579
11 Coleman, R., Finean, J. B. and Thompson, J. E. (1969) *Biochim. Biophys. Acta* 173, 51–61
12 Rodriguez de Lores Arnaiz, G., Alberici, M. and de Robertis, E. (1967) *J. Neurochem.* 14, 215–225
13 Skipski, V. P., Smolowe, A. F., Sullivan, R. C. and Barclay, M. (1965) *Biochim. Biophys. Acta* 106, 386–396
14 Skipski, V. P., Peterson, R. F. and Barclay, M. (1964) *Biochem. J.* 90, 374–378
15 King, E. J. (1932) *Biochem. J.*, 32, 292–297
16 Galliard, T., Michell, R. H. and Hawthorne, J. N. (1965) *Biochim. Biophys. Acta* 106, 551–563
17 Snyder, F. and Stephens, N. (1959) *Biochim. Biophys. Acta* 34, 244–245
18 Shohet, S. B., Nathan, D. and Karnovsky, M. L. (1968) *J. Clin. Invest.* 47, 1096–1106
19 Higgins, J. A. and Green, C. (1967) *Biochim. Biophys. Acta* 144, 211–220
20 Guder, W., Weiss, L. and Wieland, O. (1969) *Biochim. Biophys. Acta* 187, 173–185
21 Cater, B. R. and Hallinan, T. (1971) *Biochem. J.* 124, 60 P–61 P
22 Cater, B. R. and Hallinan, T. (1971) *FEBS Lett.* 16, 137–140
23 Cater, B. R., Walkden, V. and Hallinan, T. (1972) *Biochem. J.* 127, 37P–38P
24 Meldolesi, J., Jamieson, J. D. and Palade, G. E. (1971) *J. Cell. Biol.* 49, 130–149
25 Meldolesi, J., Jamieson, J. D. and Palade, G. E. (1971) *J. Cell. Biol.* 49, 150–158