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DEMONSTRATION AND CHARACTERIZATION OF PARTIAL GLYCERIDE SPECIFIC LIPASES IN PIG THYROID PLASMA MEMBRANES

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SUMMARY Purified pig thyroid plasma membranes were treated with bacterial phospholipase C. Phospholipids were hydrolyzed, producing diacylglycerols and monoacylglycerol. The products, however, decreased appreciably in the later period of incubation, suggesting the presence of lipase hydrolyzing partial glycerides in the membranes. The presence of the lipase(s) was proved by the use of exogenous substrates. In contrast to ordinary lipases, the plasma membrane enzyme did not hydrolyze triacylglycerol, but diacylglycerol. Apparent Km of the enzyme for 1,2-diacylglycerol was 1.25 mM and optimal pH, 7 - 7.5. The membranes also had the activity to hydrolyze 2-monoacylglycerol but not 1-monoacylglycerol. On the basis of these results, we proposed a scheme in which the enzyme(s) participate in arachidonic acid production from phosphatidylinositol (PI) under the influence of thyrotropin.

INTRODUCTION

Lipases which hydrolyze partial glycerides specifically have been found in rat erythrocyte membranes (1) and rat brain microsomes (2). The enzymes have been suggested to play a role in phospholipid metabolism in cell membranes. Recently, this type of lipase activity was found also in platelets and was assumed to have a role in a thrombin-induced prostaglandin synthesis as the arachidonic acid producing system (3, 4).

In our preceeding paper (5), we demonstrated TSH action on phospholipid metabolism, especially a very rapid and temporal increase in the amount of DG[1/] containing arachidonic acid. The present paper deals with evidence for the presence of lipases hydrolyzing DG or MG[1/] in the thyroid plasma membrane and some of the biochemical properties of the enzymes.

MATERIALS AND METHODS

1) Preparation of plasma membranes and its treatment with phospholipase C

Thyroid plasma membranes were prepared from fresh pig thyroid glands obtained at a local slaughterhouse, according to Suzuki et al. (6). Under an electronmicro scope the preparation was shown to be practically pure. Na, K-ATPase activity (7) as a marker enzyme for plasma membrane was 11.7 μmoles/hr/mg protein (17.5 fold over the activity of crude homogenate). The plasma membrane preparation was

1/ Abbreviations used are: MG, monoacylglycerol; DG, diacylglycerol; PI, phosphatidylinositol; BSA, Bovine Serum Albumin; TSH, thyrotropin.

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incubated in 25 mM Tris-HCl (pH 7.4) containing 0.75 mM $CaCl_2$ at 37 C with 100 μg/ml of phospholipase C (Cl. welchii) or with 15 μg/ml of PI-specific phospholipase C purified from B. cereus according to Ikezawa et al. (8).

2) Extraction and analyses of lipids Procedures were the same as described in the preceeding paper (5) except 4.8 volumes of chloroform-methanol mixture added to stop the reaction. 1-MG and 2-MG were separated by thin layer chromatography, using boric acid impregnated plates (9).

3) Determination of membrane-bound lipase activity Neutral lipids (10 μmoles/ml H_2O) were emulsified by sonication, using a 10 kHz sonicator (Ohtake Works, Tokyo, Japan) at 100 w for 5 min. This treatment resulted in producing opaque, homogeneous and stable emulsions of all neutral lipids except triolein. Triolein was emulsified by the same treatment, but in the presence of 1 % bovine serum albumin to obtain a stable emulsion.

Four tenth to 1 μmole of emulsified lipid was added into 1 ml of 25 mM Tris-HCl (pH 7.4) containing a certain amount of the plasma membranes and incubated at 37 C. At the time indicated, the reaction was stopped by the addition of 4.8 volumes of chloroform-methanol mixture (2 : 1 v/v). Lipids were analyzed in the manner described in the previous section.

4) Other chemicals and analyses 1,2-DG was prepared from egg lecithin by phospholipase C (Cl. welchii) treatment. 2-MG was isolated from a crude hydrolyzate of salad oil digested with pancreatic lipase (Sigma), by silicic acid column and thin layer chromatography. 1,3-diolein, triolein, 1-monopalmitin, 1-monoolein and ceramide were purchased from Sigma.

Phosphate was assayed by the method of Ames et al. (10) and membrane protein was solubilized with deoxycholate and analyzed by Lowry's method (11) using BSA as a standard.

RESULTS AND DISCUSSION

1) Degradation of plasma membrane phospholipids by phospholipase C

When purified pig thyroid plasma membranes were incubated with a bacterial phospholipase C (Cl. welchii), about 80 % of phospholipid of the membranes was hydrolyzed within 30 min. Analysis of the reaction products by thin layer chromatography showed the formation of 1,3-DG and MG in addition to 1,2-DG which was the direct product of the phospholipase C action (Fig. 1a). When the enzyme was not added or the plasma membranes were replaced with egg lecithin, there was no production of 1,3-DG or MG. Similar results were obtained by the use of PI-specific phospholipase C prepared from B. cereus (Fig. 1b).

Fig. 2 shows time courses of the production of metabolites. 1,2-DG reached the maximum level 10 min after the onset of incubation and decreased in the following period. 1,3-DG increased slower than 1,2-DG and the maximum amount was about one third of that of 1,2-DG. MG appeared later than DGs. These findings suggested that the plasma membranes have the activity which converts the 1,2-DG to MG and 1,3-DG. In addition, the MG decreased appreciably in the later period, suggesting the presence of MG lipase in the thyroid plasma membranes. On the basis of thin layer chromatographic analysis of the MG, the decrease in MG was suggested to be due to the degradation of 2-MG (data not shown).

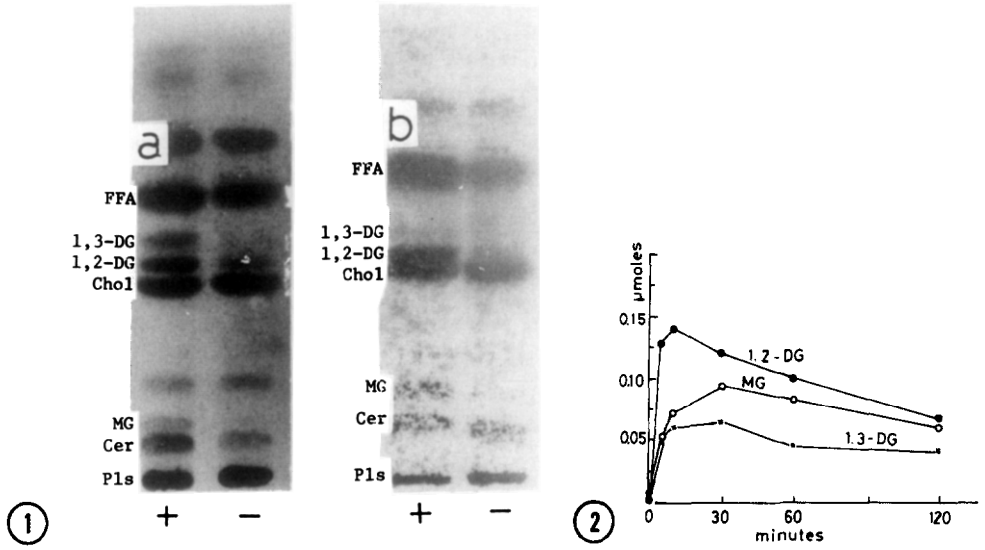


Fig. 1. Thin layer chromatography of lipids extracted from phospholipase C treated plasma membranes
(a) thyroid plasma membranes (1.6 mg of protein) treated with (+) or without (-) phospholipase C (Cl. welchii) for 60 min. (b) thyroid plasma membranes (4.9 mg of protein) treated with (+) or without (-) PI-specific phospholipase C (B. cereus) in the presence of 0.05 % deoxycholate for 30 min. The mobilities of standards of phospholipid (Pls), ceramide (Cer), MG, cholesterol (Chol), 1,2-DG, 1,3-DG and unesterified fatty acid (FFA) are indicated.

Fig. 2. The change in the lipid composition of plasma membranes incubated with phospholipase C
Thyroid plasma membranes (1.6 mg of protein) were treated with 100 μg/ml phospholipase C (Cl. welchii) under the condition described in the text.

2) Characterization of membrane-bound diacylglycerol lipase

To confirm the presence of DG lipase, 1,2-DG prepared from egg lecithin was added as a exogenous substrate, and the degradation of the DG by the enzyme of thyroid plasma membranes was examined. In this case, endogenous substrates derived from membrane phospholipids were negligible. As shown in Fig. 3, 1,2-DG was hydrolyzed linearly for at least 60 min and an equivalent amount of MG was produced concomitantly. However, we did not find any formation of 1,3-DG. This is inconsistent with the results obtained with endogenous substrate shown in Fig. Such a discrepancy cannot be explained at the present stage, although differences in substrate species may be the cause.

The DG lipase activity was abolished by the treatment of membranes with pronase and by heating at 100 C for 2 min, and when the membranes were treated with trypsin the activity decreased by 60 %.

The degradation of 1,2-DG (1 μmole/ml) increased linearly with the increase in the concentration of membranes up to 0.6 mg protein/ml (Fig. 4a). At the

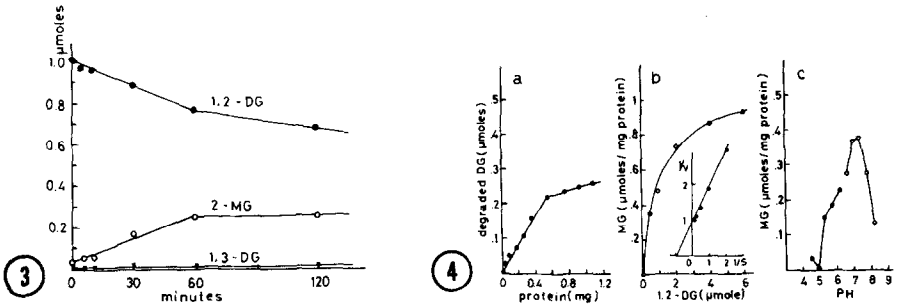


Fig. 3. Time course of hydrolysis of exogeneous 1,2-DG by membrane-bound lipase
1,2-DG (1 μmole/ml) was incubated with plasma membranes (0.57 mg of protein) under the condition described in the text.

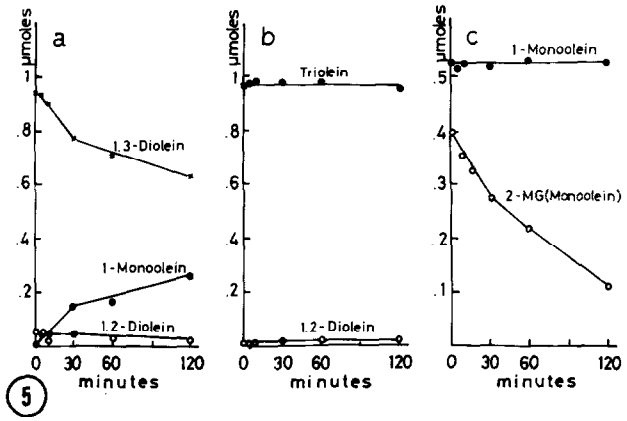
Fig. 4. Properties of membrane-bound DG lipase
(a) effect of membrane concentration on the hydrolysis of 1,2-DG (1 μmole/ml), incubation for 60 min. (b) effect of substrate concentration on DG lipase (0.2 mg of membrane protein), incubation for 60 min. (c) effect of pH on the hydrolysis of 1,2-DG. 1,2-DG (1 μmole/ml) was incubated with plasma membranes (0.2 mg of protein) for 60 min. (●-●) acetate buffer, (o-o) imidazole-HCl buffer.

membrane concentration of 0.2 mg protein/ml, at which equimolar MG was produced from DG, the reaction velocity measured by MG production showed Michaelis-Menten type saturation kinetics. Apparent Km was 1.25 mM and Vmax was 1.11 μmole/min/mg protein (Fig. 4b). pH optimum was between 7 and 7.5 (Fig. 4c). These properties of thyroid enzyme are similar to those of membrane-bound lipase of nerve ending (12). Ca^{++} (up to 2 mM) and deoxycholate (up to 1 %) did not affect the reaction.

Substrate specificity of the thyroid enzyme was investigated with synthetic substrates. Fig. 5 shows that, in addition to 1,2-DG, 1,3-diolein was hydrolyzed, producing equivalent amounts of 1-MG but triolein emulsified with BSA was not hydrolyzed.

3) Demonstration of monoacylglycerol lipase activity of the plasma membranes

The time course study shown in Fig. 1 suggested the presence of MG lipase in the plasma membranes. On the other hand, as described in the former section, MG as a hydrolytic product was not hydrolyzed appreciably under the condition for DG lipase assay. Therefore, we examined MG-hydrolyzing activity of the plasma membranes, using various MG preparations and a higher concentration of plasma membranes. As shown in Fig. 5c, 2-MG (mostly 2-monoolein) prepared from salad oil was hydrolyzed as well as DGs, while 1-monoolein was not hydrolyzed at all. 1-monopalmitin also was not degraded. These results indicated that the thyroid plasma membranes had MG lipase which was specific to 2-isomer. This type of specificity does not seem to be the only case, since MG lipase of adipocytes has been reported to be specific to 2-isomer (13).



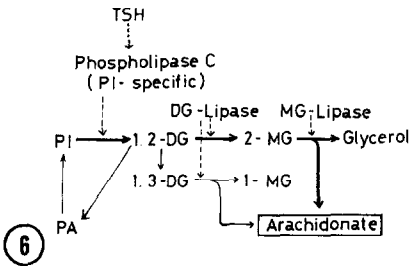


Fig. 5. Substrate specificity of membrane-bound lipase
(a) 1,3-diolein. (b) triolein. (c) 1-monoolein and 2-MG (mostly 2-monoolein). Each lipid was dispersed and incubated with plasma membranes (0.54 mg of protein) as described in the text.

Fig. 6. Proposed pathway for arachidonic acid release from membrane PI of TSH-stimulated thyroid
PI, phosphatidylinositol; PA, phosphatidic acid; DG, diacylglycerol; MG, monoacylglycerol.

At this stage of the study, we could not determine whether MG lipase is different from DG lipase or actually the same enzyme as DG lipase and has the ability to hydrolyze 2-MG too. On the other hand, the difference in stereospecificity with the substrate suggests that MG is hydrolyzed by a different enzyme from DG lipase.

All the findings in the present study are consistent with the hypothesis in that TSH stimulates PI turnover and, in the same reaction, stimulates the transient accumulation of arachidonic acid which may be a precursor of prostaglandin in the next step. On the basis of these findings, we propose the following scheme for the arachidonic acid release from membrane PI of TSH-stimulated thyroid (Fig. 6).

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