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Activation of Detergent-Solubilized Diacylglycerol Acyltransferase by Anionic Phospholipids

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Diacylglycerol acyltransferase (DGAT), which catalyzes the final step in triacylglycerol (TG) biosynthesis, is crucial for lipid accumulation and formation of lipid bodies in an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. Since solubilization of DGAT in the lipid body fraction from this fungus with 0.5% Triton X-100 gave very low recovery of the activity, some activation factors for solubilized DGAT activity were investigated. Addition of phospholipids, especially anionic phospholipids such as phosphatidic acid and phosphatidylserine, to the assay mixture greatly increased DGAT activity. The activation by these phospholipids was most prominent when 0.2% Triton X-100 was added to the assay mixture. The effect of phosphatidic acid was reproduced using DGAT fraction obtained by 0.5 M KCl elution on Mono S column chromatography. The results provide new insight on activation of DGAT during TG accumulation as well as optimal DGAT assay conditions for solubilized fractions.

Key words: diacylglycerol, diacylglycerol acyltransferase, lipid bodies, phosphatidic acid, phosphatidylserine.

Diacylglycerol acyltransferase (DGAT) [EC 2.3.1.20] operates to synthesize triacylglycerol (TG) as an energy reservoir. Regulation of DGAT is of particular interest, because this enzyme is located at a branching point for the synthesis of TG or phospholipids from diacylglycerol (DG) (*1, 2*). Although solubilization and further purification of DGAT have been reported (*3-8*), characterization of the enzyme has been hampered by its instability after detergent solubilization from membranes. Anionic phospholipids are known to activate enzymes which interact with DG, such as protein kinase C (*9*), DG kinase (*10*), and monogalactosyldiacylglycerol synthase (*11*). Thus, these anionic phospholipids might also activate DGAT activity. Although microsomal phospholipids have already been reported to activate DGAT in rat liver (*4*), the effect of individual phospholipid classes remains unclear. We have been studying DGAT to elucidate the mechanism of TG accumulation in an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. In the present study, we found that several phospholipids increased DGAT activity in the Triton X-100 extract from the lipid body fraction of this fungus. Phosphatidic acid (PA) was most potent for the activation of detergent-solubilized DGAT activity.

MATERIALS AND METHODS

Materials—[1-^{14}C]Oleoyl-CoA (58 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA). Phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), phosphatidylserine (PS) (beef brain), phosphatidylinositol (pig liver), PA (from egg lecithin), cardiolipin (beef heart), lysophosphatidylcholine (pig liver), lysophosphatidylserine (beef brain), 1-monoolein, and triolein were from Serdary Research Laboratories (Ontario, Canada). Oleoyl-CoA, lysophosphatidic acid (oleoyl), and 1,2-diolein were purchased from Sigma Chemicals (St. Louis, MO, USA). Triton X-100 was from Nacalai Tesque (Kyoto). Silica gel G TLC plates were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Solubilization of DGAT Activity in the Lipid Body Fraction—The lipid body fraction was obtained by the floatation method from fungal cells [M. *ramanniana* var. *angulispora* (IFO 8187)] which had been cultured for 4 days, as described before (*12*). The lipid body fraction was solubilized by adding an equal volume of 10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 1% Triton X-100 (final 0.5%), and incubating the mixture for 1 h at 4°C. After the incubation, the sucrose concentration of the mixture was adjusted to 0.4 M, upon which 10 mM phosphate buffer (pH 7.0) containing 0.15 M KCl, 0.3 M sucrose was overlayered. The discontinuous sucrose gradient was centrifuged at 58,000 × *g* for 3 h and the remaining soluble fraction served as the detergent extract.

Assay for DGAT Activity—DGAT activity after the detergent solubilization was measured in a reaction mixture which contained 3.4 μM (0.2 μCi/ml) [1-^{14}C]oleoyl-CoA, 1 mM 1,2-diolein, various concentrations of Triton X-100, other exogenous lipids, and enzyme sources [0.30 μg protein for the Triton X-100 extract and 0.12 μg protein for the fraction partially purified with Mono S (see below)]. The reaction was carried out at 30°C for 5 min and DGAT activity was calculated as previously described (*12*).

Abbreviations: DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; PA, phosphatidic acid; PS, phosphatidylserine; TG, triacylglycerol.

Other Methods—A Mono S column (1 ml) was equilibrated with 10 mM phosphate buffer (pH 6.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% ethyleneglycol, and 0.1% Triton X-100. The 0.5% Triton X-100 extract which had been dialyzed against the equilibrium buffer was applied to the Mono S column, and DGAT activity was eluted with the equilibrium buffer containing 0.5 M KCl. Phospholipid concentrations were determined by phosphorus analysis as described (*13*). Protein was measured by the method of Bradford (*14*).

RESULTS AND DISCUSSION

We investigated efficient solubilization of DGAT in the lipid body fraction with detergents. Although Triton X-100 most efficiently solubilized DGAT activity in the lipid body fraction, recovery of the DGAT activity in the Triton X-100 extract was quite low. To test whether some lipid(s) necessary for maintaining DGAT activity had been lost during the solubilization, several lipids were added to the DGAT assay mixture for 0.5% Triton X-100 extract. Preliminary studies showed that PA or PS activated DGAT activity in the 0.5% Triton X-100 extract. The activation by phospholipids was also affected by the Triton X-100 concentration in the assay mixture (Fig. 1). Optimal activation was obtained when 0.2% Triton X-100 was added (final Triton X-100 concentration was 0.3%, including Triton X-100 derived from the enzyme source). The concentration was higher than that (0.1%) added in the DGAT assay mixtures for the lipid body fraction and the membrane fraction (*12, 15*). The result may reflect the vesicle structure composed of DG, phospholipids and Triton X-100, which could be affected by Triton X-100 concentration (*16*). Most other phospholipids also increased DGAT activity, whereas neutral lipids had no stimulatory effect (Fig. 2). Cardiolipin as well as PA and PS greatly increased DGAT activity, but phosphatidylinositol had no effect, indicating that negative charge is not enough to account for the activation.

Although microsomal phospholipids were reported to increase DGAT activity in rat liver (*4*), the effect of anionic phospholipids on DGAT activity has not been reported yet. Recently, almost the same effect of anionic phospholipids on hepatic monoacylglycerol acyltransferase was reported (*17*). It would be quite interesting if both acyltransferases for TG biosynthesis were physiologically regulated by similar cofactors, especially PA. The activation of DGAT by anionic phospholipids also provides a new instance where phospholipids and DG interact with enzymes; DG kinase (*18*) and protein kinase C (*19*) are activated by anionic phospholipids in the presence of Ca^{2+}, whereas DG choline- and ethanolaminephosphotransferases are activated by phosphatidylcholine rather than anionic phospholipids (*20*).

The activation of DGAT by PA or PS in the fungus was not inhibited by addition of EDTA (Fig. 2), suggesting that Ca^{2+} or other metal ions are not involved in the activation

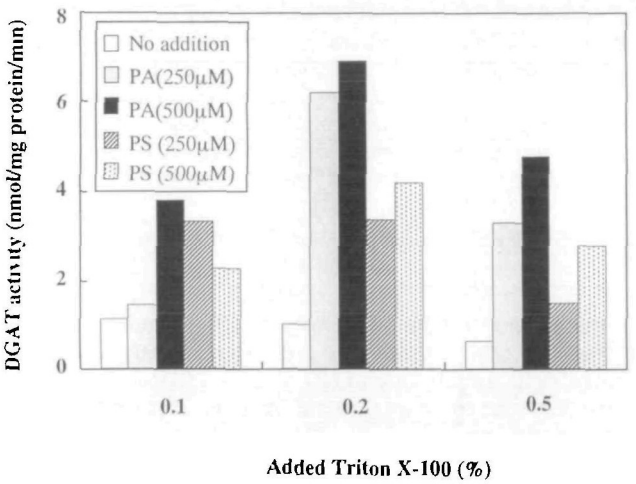

Fig. 1 **Effect of phosphatidic acid (PA) and phosphatidylserine (PS) on diacylglycerol acyltransferase (DGAT) activity solubilized from the lipid body fraction.** The enzyme was solubilized by 0.5% Triton X-100 and assayed as described in "MATERIALS AND METHODS." PA or PS together with Triton X-100 was added to the assay mixture as indicated. Triton X-100 derived from the solubilized enzyme fraction contributed an additional 0.1% to the assay mixture. Data are presented as means of duplicates for a typical experiment from among several independent ones.

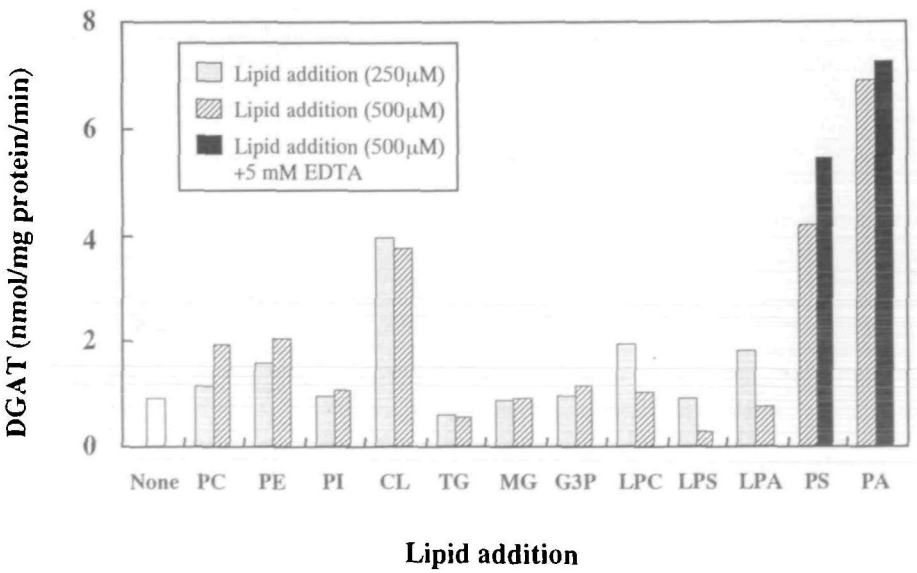

Fig. 2. **Effect of various lipids on diacylglycerol acyltransferase (DGAT) activity solubilized from the lipid body fraction.** The enzyme was solubilized by 0.5% Triton X-100 and assayed with addition of 0.2% Triton X-100 as described in "MATERIALS AND METHODS." Data are presented as means of duplicates for a typical experiment from among several independent ones. PC, phosphatidylcholine, PE, phosphatidylethanolamine; PI, phosphatidylinositol; CL, cardiolipin; TG, triacylglycerol; MG, monoacylglycerol, G3P, glycerol-3-phosphate; LPC, lysophosphatidylcholine; LPS, lysophosphatidylserine, LPA, lysophosphatidic acid; PS, phosphatidylserine; PA, phosphatidic acid.

by anionic phospholipids. Lysophospholipids showed activation of DGAT activity in the lipid body fraction at relatively lower concentrations (Fig. 2), which is in agreement with a previous observation on rat liver DGAT (*21*). Lysophosphatidic acid and lysophosphatidylserine increased DGAT activity, but not as much as PA and PS. Since the purity of each phospholipid was checked by TLC, it is unlikely that low concentrations of contaminating lysophospholipids are responsible for the effects of PA and PS.

Autoradiography of TLC plates separating reaction products of DGAT assay showed that no synthesis other than TG synthesis from [1-^{14}C]oleoyl-CoA was markedly stimulated by PA (Fig. 3). This suggests that the activation of DGAT by PA is due to a direct effect on DGAT. The possibility that a decrease in the breakdown of [1-^{14}C]-oleoyl-CoA by PA caused an apparent increase in ^{14}C-incorporation into TG can be excluded, because the amount of total [1-^{14}C]oleoyl-CoA was almost unchanged by PA. The addition of PA also increased partition of [1-^{14}C]oleoyl-CoA into the organic phase, and this also happened in the absence of enzyme sources. This may be due to direct interaction between PA and acyl-CoA.

DG dependency of DGAT in the Triton X-100 extract was obtained in the presence of PA (Fig. 4A). Since almost no DGAT activity could be detected without exogenous DG in the presence of PA, PA itself would not be hydrolyzed to DG to serve as a substrate for DGAT. Substrate saturation was reached at about 500 μM, which was lower than the value of 1,500 μM obtained from DGAT assay without PA in the lipid body fraction (*12*). The DGAT fraction obtained from Mono S chromatography (see "MATERIALS AND METHODS") showed similar DG dependency until about 500 μM, suggesting that PA activated DGAT irrespective of its purification stage. Since higher concentrations of DG rather inhibited partially purified DGAT activity (Fig. 4A), the DG concentration in the assay mixture was changed to 500 μM thereafter. Figure 4B shows the effect of PA concentration on DGAT in the Triton X-100 extract when assayed in the presence of 500 μM DG. Half-maximal activity of DGAT occurred at around 50 μM PA. This concentration of PA can be expressed as 1.0 mol % in Triton X-100 mixed micelles (calculated from total Triton X-100 concentration in the assay as 0.3% and Triton X-100 aggregation number as 140), which indicates that DGAT was activated by small numbers of PA molecules (*10*). Similar PA dependency of

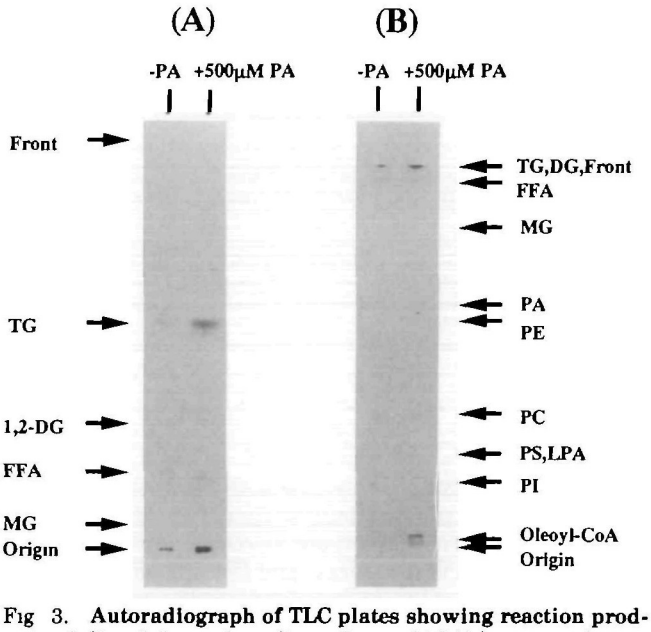

Fig 3. **Autoradiograph of TLC plates showing reaction products of diacylglycerol acyltransferase (DGAT) assay.** The enzyme was solubilized by 0.5% Triton X-100 and assayed with addition of 0 2% Triton X-100 as described in "MATERIALS AND METHODS." Reaction products of DGAT assay with or without 500 μM phosphatidic acid (PA) were analyzed by TLC developed with benzene/diethyl ether/ethanol/NH_3 (50 · 40 . 2 . 0.5) as the first solvent and hexane/diethyl ether (94 : 6) as the second solvent for separation of neutral lipids (A), and developed with chloroform/acetone/methanol/acetic acid/H_2O (50 · 20 10 10 5) for separation of polar lipids (B). FFA, free fatty acid Other abbreviations are as indicated in the legend to Fig. 2.

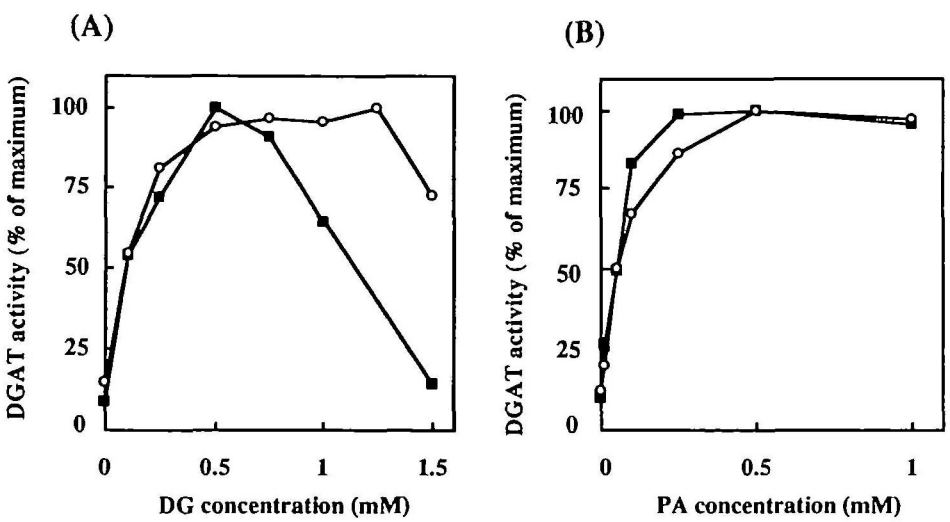

Fig 4 **Dependence of solubilized DGAT activity on the concentrations of diacylglycerol (DG) and phosphatidic acid (PA).** The enzyme was solubilized in 0.5% Triton X-100 and partially purified with Mono S as described in "MATERIALS AND METHODS." DGAT activity in the 0.5% Triton X-100 extract (○) or the Mono S column-treated fraction (■) was assayed with varying concentrations of DG in the presence of 500 μM PA (A) and with varying concentrations of PA in the presence of 500 μM DG (B). The maximum DGAT activities for the Triton X-100 extract in (A) and (B) were 6 2 and 5.8 nmol/mg protein/min, respectively, and those in the partially purified fraction with Mono S in (A), (B) were 8.7 and 8.5 nmol/mg protein/min, respectively. Data are presented as means of duplicates for a typical experiment from among several independent ones.

DGAT was obtained for the partially purified fraction (Fig. 4B).

The present study has demonstrated activation of detergent-solubilized DGAT in the lipid body fraction by anionic phospholipids, especially PA. Since the activation of DGAT by PA was also observed in the partially purified fraction, DGAT assay in the presence of PA will be helpful for further purification of DGAT from the lipid body fraction.

The results also provide interesting insight into the regulation of DGAT, which could affect the amount of TG accumulation and the channeling of DG into TG or phospholipids. DGAT solubilized from the membrane fraction in the fungus as well as from the lipid body fraction was activated by PA (data not shown), suggesting that DGAT in both lipid bodies and membranes could be activated by PA or other anionic phospholipids. Although the amount of PA under normal culture conditions was only a trace (*12, 15*), PA is an important intermediate for glycerolipid biosynthesis in this fungus; PA was extensively labeled by incorporated ^{14}C-fatty acids when *de novo* lipid biosynthesis was modulated by trifluoperazine (*22*). In addition to PA, the fungus contained a small amount of PS, which represented around 0.5% of total lipids at the stationary phase (*15*). Further studies on the distribution and metabolism of anionic phospholipids are required to help establish the physiological importance of the *in vitro* activation of DGAT by these anionic phospholipids.

Whereas DGAT is activated by anionic phospholipids, DG cholinephosphotransferase and DG ethanolaminephosphotransferase are known to be activated by phosphatidylcholine (*20*). The preference for phospholipids among DG-utilizing enzymes may cause differential activation of these enzymes. It has been reported that DGAT is regulated by a phosphorylation-dephosphorylation mechanism (*23*), by Mg^{2+} concentration (*24*) and by phospholipids surrounding the enzyme (*25*). Although further studies are required, the present results raise the possibility that the channeling of DG into TG or phospholipids is regulated by phospholipids surrounding DG-utilizing enzymes.

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