Utilization of membranous lipid substrates by membrane-bound enzymes

Intramembrane and intermembrane hydrolysis of diacylglycerol by lipase of rat liver microsomes

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The membranous lipase of rat liver microsomes was used to hydrolyze diacylglycerol (DG), generated within the microsomal membrane by treatment with phospholipase C, in two separate interactions. For an intramembrane enzyme-substrate interaction, the enzyme and DG were present in the same microsomes. For intermembrane interactions, native microsomes of rat liver were used as carriers of the enzyme, while heated and phospholipase C-treated microsomes of rat liver or brain were employed as carriers of the substrate. The *v vs S* curves of the intermembrane interaction were hyperbolic while those of the intramembrane utilization were parabolic.

Diacylglycerol *Phospholipase C* *Rat liver microsome* *Membrane-bound enzyme*

1. INTRODUCTION

To ascertain that in the intermembrane interaction, DG was not transferred from the substrate-containing to the enzyme-carrying membrane, rough endoplasmic reticulum of rat liver (carrying the lipase) was incubated with [^{3}H]DG-containing brain microsomes as the source of the substrate. Subsequent separation in a sucrose-CsCl gradient provided liver rough endoplasmic reticulum devoid of radioactivity, thereby suggesting that in the course of the intermembrane interaction, the substrate (i.e., [^{3}H]DG) indeed remained in its original membranous carrier, the brain microsomes.

Previous communications from this laboratory described the hydrolysis of diacylglycerol (DG), generated in microsomes of rat brain by a lipase residing in these membranes [1]. Hydrolysis of this membranous substrate was followed using enzymes present in the same membranes (intramembrane interaction) or in external membranes (intermembrane interaction [2]. Because of the acidic pH-optimum of the lipase, the reaction was conducted at pH 4.8. At this pH the microsomes flocculated and during the incubation sedimented to the bottom of the reaction tube, thereby restricting the motion of the microsomes. The possibility was considered that the flocculation at pH 4.8 induced a non-physiological proximity which facilitates the intermembrane interaction, either by direct contact between the membranes or by transfer of substrate (diacylglycerol) to the enzyme-containing membrane. To obviate this possibility studies, described here, were done using microsomes of rat liver whose lipase hydrolyses tri- and diacylglycerol at a neutral or slightly alkaline pH. At this pH, the microsomes did not flocculate and remained suspended throughout the incubation period. Nevertheless they hydrolysed diacylglycerol by the intermembrane as well as intramembrane routes. This suggested that the previous results observed using the brain microsomes [2] were also not a consequence of the flocculation, induced by the acidic

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pH of the reaction. To obviate the possibility that the intermembrane utilization occurs by transfer of DG from the substrate-containing to the enzyme-containing membrane, liver microsomes were used as source of enzyme and brain microsomes as source of the DG-substrate. They were interacted at pH 7.4 and then separated in a density gradient. The data proved that there was no transfer of DG from the brain to the liver microsomes in the course of the intermembrane interaction of the two particles.

2. MATERIALS AND METHODS

[9,10-$^3H_2$]Palmitic and [9,10 -$^3H_2$]oleic acids were purchased from Amersham Radiochemical Center and diluted with the corresponding non-radioactive fatty acids to about 200 μCi/μmol. Phospholipase C (type-I, from *Clostridium perfingens*), phospholipase A_2 (from *Crotalus admanteus*), and human serum albumin (fraction V) were purchased from Sigma.

2.1. *Preparation of microsomes*

Microsomes of rat brain were prepared in 0.32 mM sucrose, as in [3]. Microsomes of rat liver were similarly prepared except that 0.25 M sucrose was used. For labelling of the lipids of brain or liver microsomes, 18-day-old rats were injected intracerebrally [4] or intravenously into the tail veins, with 0.25 μl containing 4 μCi each of the tritium-labelled palmitic and oleic acids. After 1 h the rats were sacrificed and the microsomal fraction isolated.

2.2. *Phospholipase C treatments*

(i) Microsomes (1 mg protein) which had been heated for 10 min at 65°C to inactivate their lipase were suspended in 25 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$ and incubated with 0.1 mg of phospholipase C for 10 min at 37°C. Nine ml of cold, isotonic sucrose was added, the membranes were sedimented for 1 h at 36 000 rpm and dispersed in isotonic sucrose.

(ii) For generation of diacylglycerol in native liver microsomes, 1 mg of the latter was incubated for 1 h at 4°C with 0.1 mg of phospholipase C; 9 ml of cold 0.25 M sucrose was added and the membranes were sedimented as described above. For partial degradation of membrane phospholipids, the same procedure was followed except that smaller quantities of phospholipase C were used.

2.3. *Isolation of microsomal fractions*

Rat liver microsomes in 0.25 M sucrose (1 ml) were layered over 5 ml of 1.4 M sucrose containing 15 mM CsCl and centrifuged for 2 h at 100 000 × *g* [5]. The rough endoplasmic reticulum (RER) sedimented to the bottom of the tube. Smooth endoplasmic reticulum of rat liver was located at the interface between the 0.25 and 1.4 M sucrose layers. Microsomes of rat brain which had been heated at 65°C, then treated with phospholipase C and again sedimented and resuspended also concentrated at this same interface.

To test the separation of liver RER and brain microsomes, one of these respective organelles was radioactively-labelled by administration of ^3H-labelled fatty acids as described above. After centrifugation, the fractions on top and at the bottom of the sucrose – CsCl layer were collected and extracted as in [7], modified by adding 2% acetic acid to the methanol. The phases were separated and the lower, chloroform phase was evaporated. After chromatography on thin-layer plates of silica gel G, the lipid spots were scraped and their radioactivity determined [3]. In parallel, the protein content [9] as well as lipase activity were determined as described in the legend to fig. 1. Localization of the RER was also followed by determining the activity of cytidine diphosphocholine: 1,2-diacylglycerol choline phosphotransferase [6]. Phospholipase A_2 degradation of the labelled lipids in the microsomes was carried out as in [8].

3. RESULTS AND DISCUSSION

Eighteen-day-old rats were injected intravenously with equimolar quantities of ^3H-labelled palmitic and oleic acids. After 1 h the animals were sacrificed and the livers extracted with chloroform methanol mixtures. When these lipids were treated with snake venom phospholipase A_2 [8], about 90% of the radioactivity thereby released was located in the fatty acid moiety, suggesting that it originated mostly from the oleic acid which had been incorporated into the 2(β) position of the phospholipids. This contrasts with the experiments using rat brain where intracerebral administration

of a similar mixture of palmitic and oleic acids resulted in their incorporation into either the 1 or the 2 position of the phospholipid molecule. As a consequence of that labelling pattern, treatment of the brain microsomes with phospholipase C produced doubly-labelled DG derived from their phospholipids. In contrast, the DG obtained from the phospholipids of the liver microsomes was labelled mostly in the 2 position.

As shown in fig. 1, treatment of the radioactively-labelled microsomes of rat liver with phospholipase C for 10 min at 37°C, released about 70% of the radioactivity of the phospholipids as free fatty acids. This contrasts with parallel experiments using microsomes of rat brain, where diacylglycerol and not fatty acid was the product of phospholipase C action [1]. This suggested that in rat liver microsomes, the diacylglycerol which is produced by the phospholipase C is further hydrolysed by action of a lipase present in these organelles. In the experiment shown in fig. 1, the radioactivity of monoacylglycerol remained practically constant, but, because of the above-mentioned labelling pattern (i.e., radioactivity present mostly in the 2-position) it was impossible to assess if monoacylglycerol might have been an intermediate in the hydrolysis of DG by the diacylglycerol lipase.

The following kinetic studies were done (fig.1). Radioactively-labelled liver microsomes were treated with phospholipase C for 10 min at 4°C, sedimented at 100 000 × *g* for 1 h and dispersed in 0.25 M sucrose containing 25 mM Tris–HCl, pH 7.4. Further incubation at 37°C resulted in a decrease of the membrane-diacylglycerol with a concomitant stoichiometric release of radioactively-labelled free fatty acid. As defined in previous publications [2,10] the degradation of the membrane diacylglycerol by the membranous lipase is defined as an 'intramembrane utilization'.

Fig. 1 also has two insets, each describing the rate of hydrolysis of DG as a function of increasing concentrations of this compound in the microsomal membrane. In both insets, the upper abscissa shows the percentage of membrane phospholipids hydrolysed by phospholipase C, the lower abscissa expresses this as DG content (presented in dpm), and the ordinates record the decrease in DG. Inset A translates the data of fig. 1, i.e., the decrease of DG, as a function of time

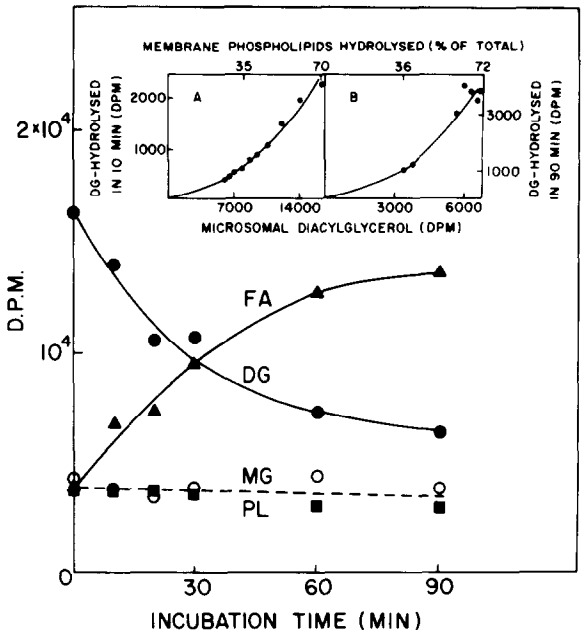

Fig. 1. Dependence of the hydrolysis of diacylglycerol in membranes containing enzyme as well as [^{3}H]diacylglycerol on the incubation time. Incubation mixtures, in a final volume of 0.5 ml, consisted of liver microsomes (2.7 mg protein) containing [^{3}H]diacylglycerol as well as lipase activity and Tris–HCl buffer, pH 7.4 (12.5 μmol). The tubes were incubated at 37°C for the times specified in the figure, terminated and analyzed as described in section 2. The estimated specific radioactivity of [^{3}H]DG was 2 × 10^4 dpm/pmol. FA, fatty acid; PL, phospholipids; MG, monacylglycerol; DG, diacylglycerol. (Inset A) Rate of hydrolysis of [^{3}H]diacylglycerol as a function of its concentration in the rat liver microsomes. The data were calculated from the main curve in fig. 1 as explained in the text. (Inset B) Dependence of hydrolysis of diacylglycerol on varying concentrations of the substrate in a fixed quantity of membranes. Tubes containing 12.5 μmol Tris–HCl (pH 7.4), 0.5 μmol of $CaCl_2$ and 1.4 mg protein of rat liver microsomes were mixed with phospholipase C, in quantities varying from 0 to 2.2 mg. After 1 h at 4°C, 9 ml of 0.25 M sucrose was added, and the tubes were centrifuged for 90 min at 100 000 × *g*. The sedimented membranes were then resuspended in 0.5 ml of 0.25 M sucrose, containing 25 mM Tris–HCl (pH 7.4) and further incubated for 90 min at 37°C.

into a *v vs S* curve. For this purpose, the rate (*v*) is defined as the decrease in DG content calculated for each 10-min period of incubation and *S*, the respective membrane-DG content in the beginning

of each 10-min period. The *v vs S* curve has a parabola-like shape. Inset B also presents a *v vs S* curve, but in this case the data stem from an independent experiment which was done as follows: fixed quantities of liver microsomes were treated with increasing concentrations of phospholipase C. After 1 h at 4°C, 9 ml of cold sucrose was added, these modified microsomes were sedimented in the cold at 100000 × *g* for 1 h and resuspended in the Tris-buffered sucrose. As a consequence of this treatment they contained increasing concentrations of membrane-DG. After adjusting temperature to 37°C, the decrease of DG content was measured following incubation for 90 min. When compared to the parallel experiment shown in inset A, the latter is a rather long incubation time. Nevertheless, the shape of the *v vs S* curve was again parabola-like and very similar to that shown in inset A. The parabolic shapes of the two insets to fig. 1 suggest activation by substrate of the intramembrane hydrolysis of diacylglycerol. This type of *v vs S* curve for intramembrane hydrolysis has already been observed in other systems [2,10].

The above data were a consequence of enzyme-substrate interaction by the intramembrane route. The following experiments test if an intermembrane interaction can also occur using the rat liver microsomes. Liver microsomes, which have lipase but not DG, were mixed with preheated and phospholipase C-treated microsomes, which therefore contained DG but not enzyme. In these experiments, microsomes of rat liver (fig. 2A) or brain (fig. 2B) were used respectively, as source of the membranous DG, and the *v vs S* curves were seemingly hyperbolic. This contrasts with the parabola-like shapes of the *v vs S* curves resulting from the intramembrane interaction (fig. 1, insets) suggesting different mechanisms of enzyme-substrate interaction in the respective inter- and intramembrane modes of utilization.

The data of fig. 2 demonstrate an intermembrane utilization of DG-containing liver or brain by enzyme-containing liver microsomes. It was expected that after incubation, each of the reaction components should still be localized in their respective membranes. Thus, the enzyme should remain in the native liver microsomes and the product (i.e., fatty acid) as well as the residual, unreacted DG in the original substrate-containing membranes.

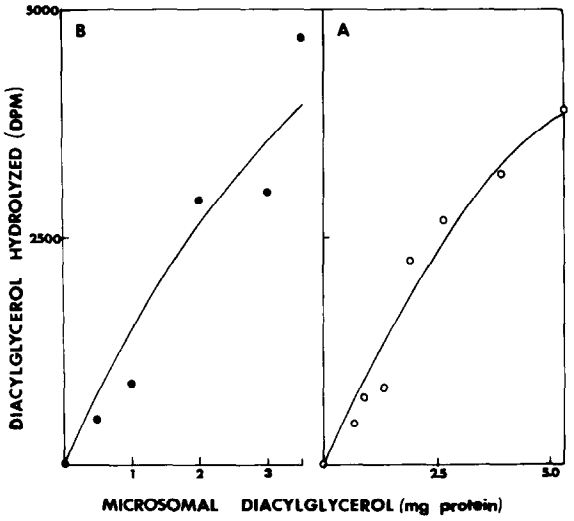

Fig. 2. (A) Dependence of the hydrolysis of [^{3}H]DG in heat-inactivated liver microsomes by lipase in native liver microsomes, by an intermembrane interaction; 1.25 mg protein of untreated rat liver microsomes which contain lipase, were mixed with varying quantities of liver microsomes containing [^{3}H]diacylglycerol whose enzyme had been heat-inactivated. Each tube, in a vol. of 0.5 ml, contained 12.5 μmol of Tris-HCl (pH 7.4) and was incubated for 90 min at 37°C. Termination and analysis are described in section 2. (B) Dependence of the hydrolysis of [^{3}H]DG in heat-inactivated brain microsomes by lipase in native liver microsomes, by an intermembrane interaction. Conditions were similar to those of A except that 1.2 mg protein of untreated liver microsomes were mixed with varying quantities of preheated and phospholipase C-treated brain microsomes, containing [^{3}H]diacylglycerol.

To test if this is indeed the case, experiments were done to separate the enzyme- and substrate-carrying membranes. Rough endoplasmic reticulum (RER) was prepared from rat liver and used as enzyme source, and microsomes of rat brain, which had been preheated and then treated with phospholipase C to generate DG, as source of substrate. When layered separately on top of 1.4 M sucrose containing 15 mM CsCl and centrifuged for 2 h at 100000 × *g*, the brain microsomes floated over the sucrose-CsCl cushion. In contrast, about 70–75% of the liver RER sedimented to the bottom of the tube, while about 25–30% floated on top of the sucrose-CsCl.

An experiment was then done in which radioactively-labelled brain microsomes which had been

treated with phospholipase C to generate [^{3}H]DG were mixed with liver RER containing the lipase. Incubation was for 90 min at 37°C and the entire reaction mixture was layered on sucrose-CsCl and centrifuged as described above. The bottom of the tube which contained the liver RER had no radioactivity whatsoever, suggesting that they were not contaminated with brain microsomes. Furthermore, this also demonstrated that there was no transfer, either of DG or of its degradation product (i.e., fatty acid), from the substrate-containing brain membranes to the enzyme-containing liver RER. Because of cross-contamination of the brain membranes by liver RER on the top of the cushion, this experiment did not permit assessment of whether lipase might have been transferred from the liver to the brain microsomes.

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