Interaction of Membranous Enzymes with Membranous Lipid Substrates

HYDROLYSIS OF DIACYLGLYCEROL BY LIPASE IN RAT BRAIN MICROSOMES

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The phospholipids in rat brain microsomes were labeled with tritium by intracerebral administration of radioactive fatty acids and converted to diacylglycerol with phospholipase C. The latter lipid was hydrolyzed in situ at pH 4.8, to monoacylglycerol and fatty acid by the endogenous microsomal lipase. This paper provides evidence for the presence of the intramembrane interaction as well. A probable difference in the mechanisms of these two interactions is suggested by different shapes of the curves that describe the reaction rate as a function of the endogenous substrate. The curve resulting from the intermembrane interaction was hyperbolic while that representing the intramembrane route was of a parabola-like shape. Competition experiments suggested that when given a choice between the two, the enzyme utilized preferentially the substrate molecules in its own membrane.

Numerous papers described the utilization of lipid substrates by their respective enzymes. In most of these, the aim was to extract, solubilize, and purify the enzyme, then incubate it with a dispersion of the pure lipid substrate (see Refs. 1 and 2 for reviews and Ref. 3 for the proceedings of a recent conference on Enzymes of Lipid Metabolism). In cells of living organisms, the vast majority of lipids occurs in membranes and many of the enzymes that utilize lipids are also membrane-bound. Therefore, most enzyme-catalyzed reactions in the cell of a living organism will involve action of an enzyme in a membrane on a lipid substrate in a membrane. The mode of such interaction and utilization is, however, very poorly, understood to date and precise mechanisms are lacking. Furthermore, an experimental approach has not been developed that would clearly define if enzymatic utilization of the lipid substrate occurs when the two components are located in the same membrane or in two adjacent membranes.

This paper describes a system in which a membranous enzyme utilized a lipid generated in the membrane. For this purpose, the procedure of Cabot and Gatt (4) was followed. Microsomes of rat brain which contain lipases (5) were treated, at pH 7.4, with phospholipase C, thereby generating diacylglycerol in these membranes. Further incubation, at pH 4.8, resulted in degradation of this lipid by the microsomal lipase (4). The hydrolysis of the endogenous diacylglycerol by the membranous enzyme is the subject of this paper. Two preliminary reports were presented in abstract form (6, 7).

MATERIALS AND METHODS

[9,10-3H]Palmitic acid and [9,10-3H]oleic acid were purchased from Amersham and diluted with the corresponding nonradioactive fatty acids to about 200 μCi/μmol. Phospholipase C (Type I of Clostridium perfringens) and bovine serum albumin (Fraction V) were purchased from Sigma.

Microsomes of Rat Brain—Microsomes containing diacylglycerol lipase activity were prepared as described by Cabot and Gatt (4). For labeling the microsomal phospholipids, 18-day-old rats were injected, intracerebrally with equimolar quantities of 3H-labeled palmitic and oleic acids (each rat obtained 0.025 ml containing about 4 μCi of each of these fatty acids). The procedures were essentially those of Yau and Sun (8) and Cabot and Gatt (4). For complete inactivation of the lipase, the microsomes, suspended in 0.32 M sucrose were heated for 10 min at 65°C.

Treatment with Phospholipase C—The following procedure was used to enrich microsomes with radioactively labeled diacylglycerol. A suspension of microsomes was incubated with phospholipase C (0.1 mg/mg of microsomes) for 10 min at 37°C in a medium containing 25 mM Tris-HCl, pH 7.4, and 1 mM CaCl. The reaction was stopped by adding 1.5 μmol of EDTA, pH 7.4. When only partial degradation of the membrane phospholipids was required this procedure was repeated using smaller quantities of phospholipase C.

Assay of Diacylglycerol Lipase Activity—For testing intermembrane activity, untreated microsomes, suspended in 0.32 M sucrose, were mixed with radioactively labeled microsomes that had been heated to denature the enzyme and then treated with phospholipase C to generate endogenous diacylglycerol. After 10 min at this pH, sodium acetate buffer, pH 4.8, was added to a final concentration of 66 mM and the tubes were further incubated, in volumes of 0.5 ml for 30 min. For testing intermembrane interaction, a suspension of microsomes from rats that had been injected with radioactively labeled fatty acids and subsequently treated with phospholipase C was adjusted to pH 4.8 with sodium acetate buffer to a final concentration of 66 mM and incubated, in a volume of 0.5 ml for 30 min at 37°C. In either system the reaction was terminated by the procedure of Bligh and Dyer (9) modified by adding 2% acetic acid to the methanol. The phases were separated and the lower, chloroform phase was evaporated under an infrared lamp. The residue was dissolved in chloroform/methanol, 2:1, and aliquots were applied to thin layer plates of Silica Gel G. The plates were first developed in petroleum ether (40-60°C)/diethyl ether/acetic acid (60:40:1) and dried. A line was etched 3 cm above the origin and the plates were again developed, to the etch line in methyl ether. This procedure facilitated migration of monoacylglycerol. The lipids were visualized in iodine vapors and spots corresponding to the various lipids were scraped into scintillation vials containing 2 ml of Triton X-100/ethanol, 1:1. After mixing,
RESULTS

When treated with phospholipase C, at pH 7.4, about 70% of the phosphoglycerides of rat brain microsomes were converted to diacylglycerol. Changing the pH to 4.8 and further incubating at 37°C, resulted in degradation of the diacylglycerol to monoacylglycerol and fatty acid by the acidic lipase present in these membranes (4, 5). These experiments did not permit a conclusion as to whether a diacylglycerol molecule was utilized by an enzyme molecule residing in its own membrane or on an external, adjacent membrane. To test the latter possibility, membranes containing lipase but no diacylglycerol were mixed with membranes containing this substrate, but no enzyme (for preparation of the latter see "Materials and Methods" and legend to Fig. 1). Fig. 1 shows the hydrolysis at pH 4.8 of the endogenous [3H]diacylglycerol, in a fixed quantity of the substrate-containing membranes by increasing concentrations of enzyme-containing membranes. The figures show that the enzyme indeed interacted with and degraded the substrate which resided in a separate membrane. Furthermore, the rate of hydrolysis of the endogenous was a linear function of the quantity of the E-containing membranes.

Fig. 2 describes a similar experiment in which the concentrations of the diacylglycerol-containing membranes were varied and that of enzyme-containing membranes was constant. The results again show that the enzyme and substrate which resided in separate membranes interacted with each other. In this experiment the curve which described the rate of hydrolysis of the substrate as a function of increasing concentrations of the diacylglycerol-containing membranes was hyperbolic.

The existence of an intermembrane interaction makes it rather difficult to demonstrate directly the existence of an intramembrane utilization of endogenous diacylglycerol in membranes that contain enzyme as well as substrate. Occurrence of the latter route could be demonstrated by eliminating the intermembrane interaction. For this purpose a fixed quantity of membranes containing both enzyme and [3H]diacylglycerol were mixed with increasing quantities of heat-denatured microsomes, devoid of either enzyme or substrate ("inert" membranes). After preincubating for 10 min at pH 7.4, buffer at pH 4.8 was added and the hydrolysis of the diacylglycerol at this pH was measured. The presence of the inert membranes was expected to have no effect on the intramembrane E-S interaction, while the rate via the intermembrane route, which requires a physical contact of enzyme- and diacylglycerol-containing membranes, should be markedly reduced. Thus, if the inert membranes were to exceed the E- and S-containing membranes 20-fold, the rate of diacylglycerol hydrolysis via the intermembrane route would be reduced to only about 5% of the original value. The data of Fig. 3, Curve A, show a lesser degree of hydrolysis of diacylglycerol as a result of increasing concentrations of the inert membranes, but the lowest value reached was 40% of that observed in the absence of the inert membranes. This therefore suggested that, except for the intermembrane E-S interaction (whose existence was shown in the experiments described in Figs. 1 and 3), a second route existed, which was not affected by the presence of the inert membranes and which probably represents the intramembrane E-S interaction.

The above experiment assumed equivalence for the collision between an E- and S-containing membrane and an identical

FIG. 1. Dependence of hydrolysis of diacylglycerol on varying concentrations of enzyme-containing membranes. Membranes containing [3H]diacylglycerol whose lipase was heat-inactivated (1 mg of protein; for preparation see "Materials and Methods") were mixed with varying quantities of a suspension of untreated microsomes. Each tube contained 12.5 µmol of Tris-HCl, pH 7.4. After 10 min at 37°C, 33 µmol of sodium acetate buffer, pH 4.8, were added and the tubes, in final volumes of 0.5 ml, were incubated for 30 min at 37°C. Termination and quantitation of the reaction is described under "Materials and Methods."

FIG. 2. Dependence of hydrolysis of diacylglycerol on varying concentrations of substrate-containing membranes. Conditions were similar to those of Fig. 1, except that 0.67 mg of untreated microsomes were mixed with varying quantities of membranes containing [3H]diacylglycerol, whose enzyme had been heat-inactivated.

or an inert membrane. However, it is possible that the E- and S-containing membranes are attracted, preferentially to each other, and that this might be a reason for the incomplete inhibition of the reaction by the inert membranes. To test this, the experiment of Fig. 3, Curve A, was modified as follows: Heat-denatured microsomes were treated with phospholipase C, resulting in membranes containing nonradioactive diacylglycerol but no enzyme. Increasing concentrations of the latter were mixed, at pH 7.4, with a fixed quantity of membranes containing enzyme as well as [3H]diacylglycerol. In this case an equivalence is expected for the intermembrane interaction between a membrane containing enzyme and [3H]diacylglycerol and its identical neighbor (resulting in degradation of radioactive diacylglycerol) or a membrane containing nonradioactive diacylglycerol (whose hydrolysis will not be recorded). It was again expected that the intramembrane route should not be affected by the presence of the

1 The abbreviations used are: E, diacylglycerol lipase of rat brain microsomes with optimal activity at pH 4.8; S, substrate, diacylglycerol.
effect on the intramembrane interaction, expressed by a progressive increase in the specific activity of the enzyme.

Comparison of Curves A and B of Fig. 5 suggests that the rate of hydrolysis of diacylglycerol by the intermembrane route exceeds that resulting from the intermembrane interaction. This conclusion was further tested by mixing membranes containing [3H]diacylglycerol but no enzyme with membranes containing enzyme as well as increasing concentrations of endogenous diacylglycerol, nonradioactive or radioactive. The results of this experiment suggested that when presented with diacylglycerol molecules in the same or an adjacent membrane, the enzyme prefers for the former.

Other experiments were done to further define the properties of the membranous lipase and diacylglycerol as well as their interaction. Some of these are herewith briefly mentioned: (a) Effect of temperature. The curve which describes the hydrolysis of endogenous [3H]diacylglycerol, in membranes containing enzyme as well as substrate as a function of temperature showed two distinct transitions (Fig. 6). The first, at about 20°C most probably reflects the transition state of the lipids of the membrane. The second, at about 40°C (where the curve shows optimal activity) is probably a result of thermal instability of the enzyme. (b) The enzyme, substrate or product (monoacylglycerol) could not be solubilized by washing with sucrose or buffer, by adjusting the pH to 4.8 or by treating with phospholipase C. (c) Attempts were made to determine possible leakage of diacylglycerol from the phospholipase C-treated microsomes by mixing the latter with exogenous emulsions of diacylglycerol. These experiments were inconclusive because of adherence of the exogenous lipid to the microsomal particles. (d) Experiments were done to test the possibility that in the intermembrane interactions diacylglycerol might be transferred from the substrate-containing to the enzyme-containing membrane. For this purpose, two such membranes were mixed and then separated by density gradient centrifugation. The densities of untreated brain microsomes and those obtained after treatment with phospholipase C were similar and could not be separated. The above experiment was therefore done by mixing and then separating microsomes prepared, respectively, from rat brain and liver. 3H-Labeled microsomes of rat brain were heated for 10 min at 65°C and then treated with an excess of phospholipase C. EDTA (3 mM) was added, followed by a suspension of untreated microsomes of rat liver. After 15 min at 37°C, the suspension was layered on top of a discontinuous gradient ranging from 1.1 to 2 M of buffered sucrose (23 ml). Parallel tubes were prepared which had only the respective brain or liver microsomes. The tubes were centrifuged for 90 min at 80000 × g using a swinging bucket rotor in the MSE ultracentrifuge. The brain and some liver particles concentrated on top of the 1.45 M sucrose layer, while part of the liver microsomes floated on top of the 1.1 M sucrose layer. The latter were collected, diluted with 0.25 M sucrose, sedimented, and extracted with chloroform/methanol mixtures. The extract contained no radioactive diacylglycerol, suggesting that there was no transfer or exchange of this lipid between the two membranes.

**DISCUSSION**

Most lipids of cells of a living organism, as well as the enzymes that catalyze their biosynthesis or degradation are part of membranes. Elucidation of the mode of utilization of lipid substrates by their respective enzymes in biological membranes therefore seems imperative for understanding the reactions of lipid metabolism. This paper describes the hydrolysis of diacylglycerol, in a particulate fraction of rat brain by lipase residing in these particles. The substrate, which is normally present in these membranes was generated by splitting off in situ the polar head groups of the phospholipids with the aid of bacterial phospholipase C. The lipase, which can hydrolyze emulsions of tri- or diacylglycerol, but not monoacylglycerol (4, 10), had a pH optimum at 4.8. Subcellular fractionation of rat brain showed no enrichment whatsoever of this lipase in a lysosome-enriched fraction (4). To further rule out the possibility that the microsomal lipase might be a lysosomal enzyme that leaked out of the lysosomes and adhered to the microsomal particles, attempts were made to desorb the enzyme from the microsomes and solubilize it. These trials were not successful and provided further support to the previous conclusion (4) that the enzyme is indeed an intrinsic component of the membranes in the microsomal pellet.

The lipase-diacylglycerol system described in this paper provides a considerable flexibility of handling. The brain membranes do not contain the substrate (diacylglycerol) but have a precursor, namely the phospholipids which could be labeled by intracerebral administration of radioactive fatty acids. By adjusting the concentration of phospholipase C and the time of treatment with this enzyme the percentage of phospholipids converted in the membrane to diacylglycerol could be well controlled. The optimal pH of the enzyme is 4.8. This permitted mixing membranes containing enzyme, or substrate (as well as inert membranes, cf. Fig. 3) at pH 7.4, or both, and initiating the reaction by adjusting the pH to 4.8. Extraction and chromatographic separation on thin layer plates of silica gel permitted analysis of all components involved, namely the glycerophosphatides, neutral glycerides, and fatty acids. One should bear in mind that treating the membranes with phospholipase C converts the polar glycerol phosphatides to the less polar diacylglycerol. This results in considerable modification of the native structure of the membrane; however, several investigators have shown previously that even after degradation of about 70% of the membrane phospholipids, the bilayered structure is maintained (11-13).

Figs. 1 and 2 which describe an intermembrane E-S interaction show a linear relationship between reaction rates and
membranes containing nonradioactive diacylglycerol while the intermembrane route should be "diluted out" and reduced to about 5% when the ratio of the two types of membranes reached a value of 20:1. The experiment of Fig. 3B shows that, using these conditions, the hydrolysis of the radioactively labeled substrate was reduced to about 35% rather than the expected 5%, again suggesting the occurrence of an intramembrane E-S interaction.

Fig. 4 shows a control experiment in which increasing concentrations of membranes, containing nonradioactive diacylglycerol (and no enzyme) were added to a mixture of two separate membrane preparations, one having enzyme and the second, [3H]diacylglycerol. In this case, because of the absence of membranes containing enzyme as well as substrate only the intermembrane route exists. It was therefore expected that the diluting membranes should exert a very pronounced reduction in the reaction rates; Fig. 4 indeed demonstrates this. Thus, when the quantity of diluting membranes exceeded 10-fold that of the substrate- or enzyme-containing membranes the degradation of the radioactively labeled diacylglycerol was suppressed practically to zero. This control experiment supports the conclusions, drawn from the experiments described in Fig. 3, that an intramembrane utilization of diacylglycerol indeed occurs in the membranes containing both enzyme and substrate.

The experiment which measured the dependence of the rate of hydrolysis of endogenous [3H]diacylglycerol on increasing concentrations of this substrate (Fig. 2) used membranes that had been treated with excess phospholipase C, thereby degrading about 60 to 70% of its phospholipids. Fig. 5 shows a similar experiment in which the concentration of [3H]diacylglycerol in the membranes was increased gradually by varying the concentrations of phospholipase C. The upper abscissa of Fig. 5 shows the per cent phospholipids degraded by this enzyme, the lower abscissa translates this into [3H]-diacylglycerol content (expressed in disintegrations per min) and the ordinate shows the quantity of diacylglycerol, degraded by the endogenous lipase, at pH 4.8 in 30 min (also expressed in disintegrations per min). Fig. 5, Curve A, describes an experiment in which only the intermembrane route existed, while that shown in Fig. 5B also had an intramembrane utilization. The data of either experiment of Fig. 5 show no, or at best, very low enzymatic activity as long as the per cent phospholipids degraded by the phospholipase C was less than 25%. When more phospholipids were converted to diacylglycerol, the rates of hydrolysis of this compound increased considerably. In the experiment shown in Fig. 5, Curve A (intermembrane route only), the curve describing the rate as a function of increasing diacylglycerol was hyperbolic. In contrast, Curve B of Fig. 5 which describes an experiment also including the intramembrane route had a parabola-like shape. The latter shape suggests that increasing concentrations of diacylglycerol in the membranes had a positive cooperative
the concentration of the enzyme-containing membranes and a hyperbolic relationship to that of the diacylglycerol-containing membranes. Such curves were predicted by the Michaelis and Menten theory of an enzyme kinetics. This is of interest for two reasons. First, the above occurs between an enzymatic protein and lipid molecules that are not in true molecular solution but which are part of separate membranes or membrane vesicles. Secondly, at the acidic pH of the reaction (4.8), the microsomes flocculate and their gross motion is thereby severely restricted.

The above flocculation precluded the possibility of proving directly the existence of intramembranous utilization of diacylglycerol. Such utilization could be demonstrated theoretically by diluting a suspension of membranes to an extent that would make the intramembrane interaction statistically improbable ("infinite dilution"). Because of the flocculation of the membranes at the acidic pH of the reaction, the volume of the medium had no effect on the reaction rate, and the dilution experiment could not be performed. Therefore, an indirect approach had to be used to prove that an intramembrane route indeed existed. This utilized procedures in which the intramembrane utilization was blocked by an excess of inert membranes (Fig. 3) or was diluted isotopically by membranes containing nonradioactive diacylglycerol (Figs. 3 and 4).

The data of Fig. 5 focus attention on two interesting aspects of the utilization of the membranous lipid by the membranous enzyme. The different shapes of the two curves that describe the rate as a function of concentration of the endogenous substrate (hyperbolic for the intramembrane and paraboloidal for the intramembrane utilization) provide further support to the conclusion that these two modes of utilization indeed exist concurrently in this system. The second, striking observation is the absence of diacylglycerol hydrolysis when 75% or more of the membrane phospholipids remain intact. Finean and his co-workers (14, 15) observed the appearance of lipid microdroplets in the bilayer of phospholipase C-treated erythrocyte ghosts. Coleman et al. (16) have shown that these microdroplets appear once about 20 to 25% of the membrane phospholipids were degraded. In vitro, lipases have been shown to utilize coarse emulsions or droplets of the neutral glycerides. It is possible that the microsomal lipase can interact with the diacylglycerol only when the concentration of the molecules of this compound is large enough to form a separate domain or microdroplet in the membrane. With increasing diacylglycerol molecules in the membrane more "domains" or microdroplets are present, attracting and interacting with enzyme molecules and thereby producing the cooperative effect, in the case of the intramembrane interaction (Fig. 5, Curve B). The possibility should also be considered that in the native membrane, the acidophile lipase is latent and inoperative and becomes active only when attracted to or solubilized in a "domain" or microdroplet of diacylglycerol.

In spite of the complexity of the system, the experiments of this paper permitted an experimental approach to problems involved in utilization of a lipid substrate by an enzyme in membrane vesicles derived from a mammalian organ. More experiments using a similar approach should permit elucidation of clear-cut mechanisms of such utilization and hopefully result in better understanding of biosynthesis and degradation of the lipids in intact cells.

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