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IN VITRO EFFECTS OF CHLORPROMAZINE ON GLYCEROL-3-PHOSPHATE ACYL TRANSFERASE AND 1-ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE IN RAT LIVER MICROSOMES*

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Abstract—The *in vitro* effect of chlorpromazine on rat liver glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase was studied. Chlorpromazine decreased glycerol-3-phosphate acyltransferase activity in a concentration-dependent manner. The inhibition was competitive with respect to palmitoyl-CoA, while non-competitive with respect to *sn*-glycerol-3-phosphate. K_i was determined to be approximately 0.15 mM with respect to both palmitoyl-CoA and *sn*-glycerol-3-phosphate. From these results, together with the inhibitory effect of amphiphilic anions and neutral detergents on the enzyme demonstrated by others, it was proposed that the hydrophobic moiety of chlorpromazine competes with acyl-CoA. The activity of 1-acylglycerol-3-phosphate acyltransferase was inhibited by excess of 1-acyl-*sn*-glycerol-3-phosphate. In the acceptor concentration range where the substrate inhibition was observed, chlorpromazine showed stimulatory effect on the enzyme activity. At lower concentrations of the acceptor, however, chlorpromazine produced marked inhibition of the enzyme activity. From the kinetic analysis, the inhibition was found to be uncompetitive with respect to acyl-CoA. It was found that the enzyme was more susceptible to the inhibitory action of chlorpromazine with unsaturated acyl-CoAs than with the saturated species, raising the possibility that chlorpromazine alters the molecular species composition of phosphatidic acid produced by the acylation reaction.

Chlorpromazine and other amphiphilic cations have been shown to alter the phospholipid metabolism in various tissues [1–5]. These compounds enhance the labeling of acidic phospholipids, such as phosphatidylinositol, phosphatidic acid and phosphatidylglycerol, with [^{3}H]glycerol and [^{32}P]orthophosphate, while the synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol are concurrently depressed [1–5]. Although this redirection of glycerolipid synthesis towards acidic phospholipids was proposed to be attributed primarily to the inhibition of phosphatidate phosphatase [3], the alterations of several other enzymes in the *de novo* synthesis are also involved. Phosphatidate cytidylyltransferase [6] and inositol phosphatidyltransferase [7] could be activated with chlorpromazine under appropriate assay conditions although the activity of the former enzyme was shown not to be changed by *in vivo* application of the drug [8]. Chlorpromazine and trifluoperazine decrease the activity of phosphocholine cytidylyltransferase [9] which is thought to be a rate-limiting enzyme of the biosynthesis of phosphatidylcholine.

Of a variety of amphiphilic cations, chlorpromazine is one of the most effective compounds in altering the glycerolipid synthesis and its properties have been extensively studied. In the previous paper from this laboratory [8], the administration of chlorpromazine was shown to affect the initial step of glycerolipid biosynthesis, the acylation of *sn*-glycerol 3-phosphate, in rat liver. The objective of the present study is to investigate in detail the *in vitro* effect of chlorpromazine on glycerol-3-phosphate acyltransferase (acyl-CoA: *sn*-glycerol-3-phosphate *O*-acyltransferase, EC 2.3.1.15) and 1-acylglycerol-3-phosphate acyltransferase (acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphate *O*-acyltransferase, EC 2.3.1.51). Since it has been shown that the degree of inhibition of phosphatidate phosphatase by chlorpromazine varies depending upon the molecular species of phosphatidic acid [10], the specificity of the effect on the acyltransferases with respect to the molecular species of acyl-CoA was also investigated.

MATERIALS AND METHODS

Materials. Sodium *sn*-glycerol-3-phosphate, palmitic acid, oleic acid, linoleic acid, arachidonic acid and chlorpromazine hydrochloride were all purchased from Sigma Chemicals Co., St. Louis, MO, U.S.A. Coenzyme A was obtained from Kyowa Hakko Co., Tokyo, Japan. [1-^{14}C]Fatty acids and

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[2-^{3}H]glycerol were the products of Amersham International, Amersham, U.K. *sn*-[2-^{3}H]Glycerol-3-phosphate was synthesized enzymatically from [2-^{3}H]glycerol (500 mCi/mmol) according to the method of Chang and Kennedy [11]. Acyl-CoA were prepared by modification of Seubert's procedure [12]. 1-Acyl-*sn*-glycerol-3-phosphate was prepared from rat liver phosphatidylcholine according to the method of Long *et al.* [13].

Male Wistar rats weighing 120–150 g were used throughout the experiments. They had free access to Clea CF-2 Laboratory Chow and water prior to sacrifice.

Preparation of liver microsomes. The liver was homogenized in 4 vol of 0.25 M sucrose containing 2.5 mM EDTA and 50 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 17,000 *g* for 15 min and the supernatant was recentrifuged at the same condition in a Hitachi model 18PR-3 centrifuge. The resulting supernatant was centrifuged at 105,000 *g* for 1 hr in a Hitachi model 65P ultracentrifuge. The pellet was suspended in 20 ml of 0.25 M sucrose containing 50 mM Tris–HCl (pH 7.4) and again centrifuged at 105,000 *g* for 30 min. The pellet was finally resuspended in 5 ml of 0.25 M sucrose containing 50 mM Tris–HCl (pH 7.4) and used as a microsomal fraction.

Enzyme assays. Glycerol-3-phosphate acyltransferase activity was determined by measuring the rate of incorporation of *sn*-[^{3}H]glycerol-3-phosphate into the lipid-soluble fraction. The standard incubation mixture contained 4 mM *sn*-[^{3}H]glycerol-3-phosphate (2000 cpm/nmol), 30 μM palmitoyl-CoA, approximately 200 μg microsomal protein and 60 mM Tris–HCl (pH 7.4), in a final volume of 0.2 ml. The ingredients other than palmitoyl-CoA were preincubated at 37° for 1 min and the reaction was started by the addition of palmitoyl-CoA. After incubation at 37° for 1 min the reaction was terminated by adding 4 ml chloroform–methanol (2:1) followed by the addition of 0.8 ml of 0.2 M HCl containing 50 mM cold sodium *sn*-glycerol-3-phosphate. The tube was shaken vigorously and the phases were separated by a brief centrifugation. The lower phase was washed twice with 1.5 ml of methanol–0.1 M HCl (1:1) and transferred to a scintillation vial. The solvent was evaporated with a stream of nitrogen and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 2002. The reaction rate was proportional to the amount of enzyme within the range of 0–300 μg and to the time for the incubation period used.

In some experiments, an acyl-CoA generating system [14] was used for the determination of glycerol-3-phosphate acyltransferase activity. The incubation mixture contained 100 mM Tris–HCl (pH 7.4), 4 mM *sn*-[^{3}H]glycerol-3-phosphate, 6 mM $MgCl_2$, 0.3 mM palmitic acid, 0.3 mM linoleic acid, 0.015 mM bovine serum albumin, 20 mM KF, 3.6 mM ATP, 0.26 mM CoA, 0.5 mM dithiothreitol, and approximately 0.5 mg microsomal protein in a final volume of 0.5 ml. Before the preparation of the mixture, fatty acids were conjugated with bovine albumin as described by Ide and Weinhold [14]. Incubation was carried out at 37° for 30 min, since the reaction rate was linear at least up to 30 min. Other procedures were the same as those for the assay system where acyl-CoA was used as the substrate.

1-Acylglycerol-3-phosphate acyltransferase was assayed in the microsomes. The standard incubation mixture contained 80 mM Tris–HCl (pH 7.4), 0.06 mM 1-acyl-*sn*-glycerol-3-phosphate and approximately 40 μg microsomal protein in a final volume of 0.35 ml. After the preincubation at 25° for 1 min the reaction was started by the addition of 10–20 μM [^{14}C]acyl-CoA and incubated for another 1 min. The reaction was stopped by the addition of 6 ml chloroform–methanol (2:1) and then 1 ml 0.2 M HCl. The lower phase was washed twice with the 2 ml methanol–0.1 M HCl (1:1) and the radioactivity determined as described above. The enzyme activity was proportional to the amount of microsomal protein up to 80 μg, and to the incubation time up to 5 min.

Analytical methods. Organic phosphorus was determined by the molybdate–ascorbic acid method after digesting with 70% perchloric acid [15]. Protein was determined according to the method of Lowry *et al.* [16] with bovine serum albumin as standard.

RESULTS

Figure 1 shows the effect of chlorpromazine on the activity of microsomal glycerol-3-phosphate acyltransferase at various concentrations of palmitoyl-CoA. Since the substrate inhibition was observed at higher concentrations of palmitoyl-CoA than the 30 μM demonstrated by Zborowski and Brindley [7], the inhibition study was run in the range where the saturation curve was observed (10–24 μM). The activity of glycerol-3-phosphate acyltransferase was depressed with chlorpromazine in a concentration-dependent manner. Although the substrate concentration range employed was relatively narrow,

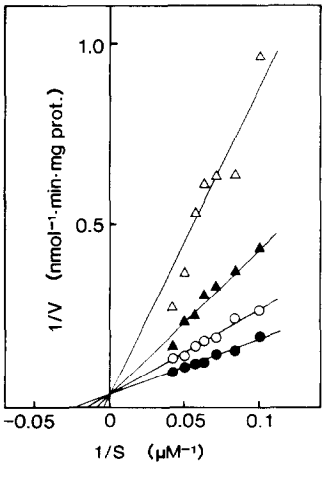

Fig. 1. Kinetic study of the inhibition of glycerol-3-phosphate acyltransferase by chlorpromazine at varying concentrations of palmitoyl-CoA. The activity was determined in the absence (●) and presence of 0.1 mM (○), 0.2 mM (▲) or 0.3 mM (△) chlorpromazine. Other conditions of assays are as described in Materials and Methods.

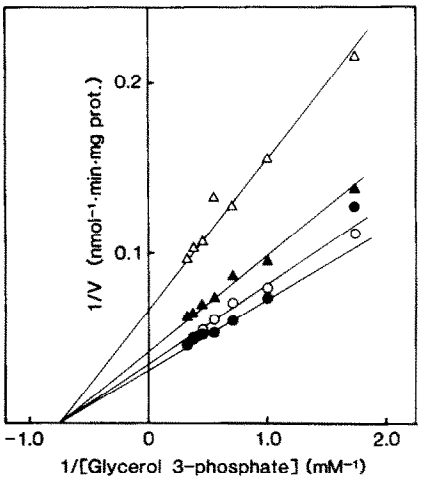

Fig. 2. Kinetic study of inhibition of glycerol-3-phosphate acyltransferase at varying concentrations of *sn*-glycerol-3-phosphate. Symbols are the same as those in Fig. 1.

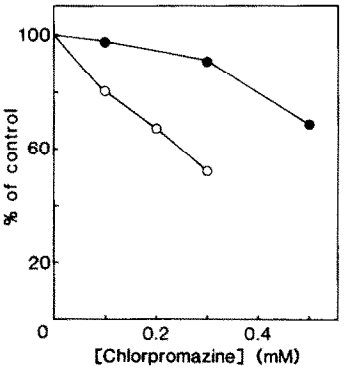

Fig. 3. Comparison of the two *in vitro* assay systems in the determination of the effect of chlorpromazine on glycerol-3-phosphate acyltransferase activity. Enzyme activity was measured either with acyl-CoA (○) or with acyl-CoA generating system (●). Assay procedure for the acyl-CoA generating system was as described in Materials and Methods. In the experiment using acyl-CoA, the incubation mixture contained 100 mM Tris–HCl (pH 7.4), 4 mM *sn*-[^{3}H]glycerol-3-phosphate, 30 μM palmitoyl-CoA, 6 mM $MgCl_2$, 20 mM KF, 0.5 mM dithiothreitol, 0.015 mM bovine serum albumin, and 528 μg of microsomal protein in a final volume of 0.35 ml. Results are expressed as the percentage of the basal activities, which were 9.43 and 4.98 nmol/min/mg protein for acyl-CoA and acyl-CoA generating system, respectively.

double reciprocal plots with or without chlorpromazine fitted straight lines which were suggestive of competitive type inhibition with respect to palmitoyl-CoA. The apparent K_m obtained in the absence of chlorpromazine was approximately 90 μM. The K_i value with respect to the acyl-donor determined from Dixon plots was approximately 0.15 mM. On the other hand, the mode of the inhibition was different for *sn*-glycerol-3-phosphate (Fig. 2). By increasing the chlorpromazine concentration, V_{max} decreased without affecting the apparent K_m (approximately 1.3 mM), indicating that the inhibition is non-competitive for *sn*-glycerol-3-phosphate. The K_i value with respect to the acceptor was determined to be approximately 0.15 mM, which was identical to that with respect to the acyl-donor.

It was demonstrated in our previous study [8] that the activity of microsomal glycerol-3-phosphate acyltransferase was not changed or even slightly increased after the administration of chlorpromazine to the rats. This discrepancy may be caused by a difference in the experimental condition between *in vivo* and *in vitro*. In order to solve this problem an acyl-CoA generating system [18] was used instead of acyl-CoA, since this system is generally thought to have closer similarity to *in vivo* conditions. It was found with an acyl-CoA generating system that glycerol-3-phosphate acyltransferase was fairly resistant to the inhibitory action of chlorpromazine (Fig. 3). No significant decrease in the enzyme activity was observed with up to 0.3 mM chlorpromazine. In the parallel experiment using acyl-CoA as acyl-donor (Fig. 3), chlorpromazine was found to be inhibitory even after addition of $MgCl_2$, KF, bovine albumin and dithiothreitol, which were included in acyl-CoA generating system, to the incubation mixture, indicating that these ingredients are not responsible for the difference in the results between the two *in vitro* assay systems. Brindley and Bowley [19] reported that 1 mM chlorpromazine was required to produce 50% inhibition in the activity of glycerol-3-phosphate acyltransferase using acyl-CoA generating system. The present result demonstrated that 0.5 mM chlorpromazine inhibited the enzyme activity by 30%, while even 0.3 mM of the drug reduced the enzyme activity to 50% in the assay system using acyl-CoA.

When 1-acylglycerol-3-phosphate acyltransferase activity was measured with 40 μg of microsomal protein at various concentrations of 1-acyl-*sn*-glycerol-3-phosphate (Fig. 4), maximal activity was obtained

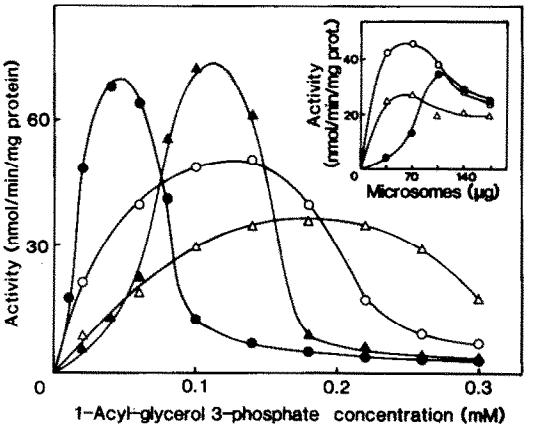

Fig. 4. Effect on chlorpromazine and Ca^{2+} ion on 1-acyl-glycerol-3-phosphate acyltransferase activity at various concentrations of the acceptor. The activity was determined with 40 μg of microsomal protein in the absence (●) and presence of 0.1 mM (○) or 0.2 mM (△) chlorpromazine, or in the presence of 50 μM Ca^{2+} (▲). Inset shows the effect of varying the amount of enzyme on the reaction rate. The concentration of 1-acyl-*sn*-glycerol 3-phosphate was 0.17 mM.

at an acceptor concentration of approximately 0.05 mM in the absence of chlorpromazine. At higher acceptor concentrations the enzyme activity sharply decreased and reached only 10% of the maximal value at the concentration of 0.17 mM. This substrate inhibition seems to be due to the decreased ratio of protein/1-acyl-*sn*-glycerol-3-phosphate, since the enzyme activity was enhanced by increasing the amount of protein at fixed concentration (0.17 mM) of the acceptor (Fig. 4, inset). Chlorpromazine enhanced the enzyme activity at low concentrations of protein (Fig. 4, inset) probably because the effective concentration of 1-acyl-*sn*-glycerol-3-phosphate decreased by binding to chlorpromazine and consequently the ratio of protein/1-acyl-*sn*-glycerol-3-phosphate increased. On the other hand, the drug exhibited inhibitory effect at high protein concentrations. When the amount of protein was fixed at 40 μg and the acceptor concentration was varied (Fig. 4), the addition of 0.1 mM chlorpromazine reduced the maximal activity of the enzyme to 70% of that of control and the optimum concentration of 1-acyl-*sn*-glycerol-3-phosphate shifted from 0.05 to 0.13 mM. Further shift of the optimum acceptor concentration (Fig. 4) was observed by increasing the chlorpromazine concentration to 0.2 mM, and the maximal enzyme activity reached 50% of that of control.

In the course of this experiment, a trace amount of Ca^{2+} was found to reduce 1-acylglycerol-3-phosphate acyltransferase activity at a low concentration of 1-acyl-*sn*-glycerol-3-phosphate. While 100 μM Mg^{2+} and K^+ did not affect the maximal activity obtained with 0.05 mM 1-acyl-*sn*-glycerol-3-phosphate and 40 μg of enzyme protein, the activity was reduced to 50% in the presence of approximately 45 μM Ca^{2+}. The effect of Ca^{2+} on the enzyme activity at varying concentration of 1-acyl-*sn*-glycerol-3-phosphate was shown in Fig. 4. Although Ca^{2+} transferred the optimum concentration of 1-acyl-*sn*-glycerol-3-phosphate similarly to chlorpromazine, the maximal activity of the enzyme remained unchanged. It is likely that Ca^{2+} decrease the effective concentration

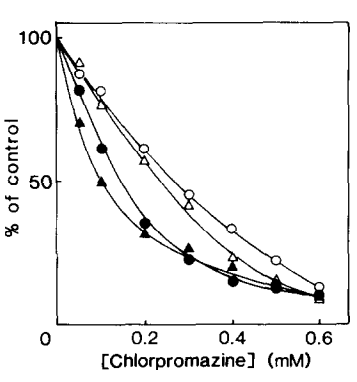

Fig. 5. Inhibition of 1-acylglycerol-3-phosphate acyltransferase by chlorpromazine. Palmitoyl-CoA (○), stearoyl-CoA (△), oleoyl-CoA (▲) or linoleoyl-CoA (●) was used as acyl-donor. Results are expressed as the percentage of the basal activities, which were 43.1, 19.3, 36.0 and 29.8 nmol/min/mg protein for palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA and linoleoyl-CoA, respectively.

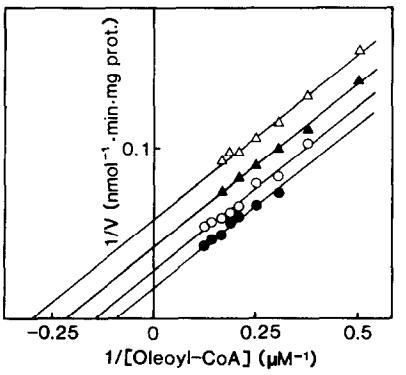

Fig. 6. Kinetic analysis of the effect of chlorpromazine on the rate of 1-acylglycerol-3-phosphate acyltransferase at varying concentrations of oleoyl-CoA. The enzyme activity was determined in the absence (●) and presence of 0.05 mM (○), 0.10 mM (▲) or 0.15 mM (△) chlorpromazine.

of 1-acyl-*sn*-glycerol-3-phosphate by binding to the substrate. In this context Ca^{2+} and chlorpromazine may share the same mechanism in shifting the optimum acceptor concentration. However, the latter seems to exert additional effect on the enzyme reaction, i.e. the reduction of maximal activity, possibly through binding to the enzyme.

The acylation of 1-acyl-*sn*-glycerol-3-phosphate with unsaturated acyl-CoAs seems to be more susceptible to chlorpromazine than with saturated acyl-CoAs (Fig. 5). The chlorpromazine concentrations required to cause 50% inhibition were approximately 0.27 and 0.23 mM for palmitoyl-CoA and stearoyl-CoA, respectively, while those values for unsaturated species, oleoyl-CoA and linoleoyl-CoA were 0.10 and 0.14 mM, respectively. The conclusion was supported by the K_i values obtained in the experiment described below. Figure 6 shows the inhibition kinetics of 1-acylglycerol-3-phosphate acyltransferase by chlorpromazine using oleoyl-CoA as an acyl-donor. Apparent K_m and V_{max} in the absence of the inhibitor were approximately 10 μM and 50 nmol/min/mg protein, respectively. A parallel decrease in both kinetic parameters were observed by the addition of increasing amounts of chlorpromazine, showing that the mode of the inhibition is of uncompetitive type. The K_i value obtained from Dixon plots was 0.080 mM. Similar kinetic profiles were obtained with linoleoyl-CoA and palmitoyl-CoA (not shown), K_i values being 0.075 and 0.170 mM for linoleoyl-CoA and palmitoyl-CoA, respectively.

DISCUSSION

Glycerol-3-phosphate acyltransferase was inhibited by chlorpromazine when assayed with palmitoyl-CoA as acyl-donor (Fig. 1). The mode of the inhibition was suggested to be competitive with respect to acyl-CoA, although it seemed to be non-competitive with respect to *sn*-glycerol-3-phosphate (Figs 1 and 2). This probably means that the hydrophobic moiety of chlorpromazine rather than the positively charged group competes with acyl-CoA,

since it has been shown that amphiphilic anion or neutral detergent can also inhibit glycerol-3-phosphate acyltransferase [19].

The activity of 1-acylglycerol-3-phosphate acyltransferase was sharply decreased at 1-acyl-*sn*-glycerol-3-phosphate concentrations higher than 50 μM in the absence of chlorpromazine. The substrate inhibition caused by the acceptor seems to be due to the formation of micelles. It has been proposed that only the free monomers of 1-acyl-*sn*-glycerol-3-phosphate can be the substrate of 1-acylglycerol-3-phosphate acyltransferase [20]. In the present experiment, the critical micellar concentration of the substrate is assumed to be approximately 0.05 mM in the assay condition used, since the maximal activity of 1-acylglycerol-3-phosphate acyltransferase was observed at that concentration of substrate. The addition of Ca^{2+} ions resulted in apparent increase in the optimum concentration of 1-acyl-*sn*-glycerol-3-phosphate to 0.11 mM without changing the maximal enzyme activity. This seems to be due to the decrease in the effective concentration of the substrate. The addition of chlorpromazine also caused the apparent shift of optimum concentration of 1-acyl-*sn*-glycerol-3-phosphate. Therefore, it is likely that chlorpromazine binds to the substrate resulting in the decrease in effective concentration. The maximal activity also decreased in proportion to the concentration of the inhibitor, indicating that chlorpromazine interacts with the enzyme. However, the mode of inhibition could not be determined with respect to 1-acyl-*sn*-glycerol-3-phosphate, because typical saturation curve was obtained only in the short range of substrate concentration in the absence of the inhibitor.

Inhibition of 1-acylglycerol-3-phosphate acyltransferase by chlorpromazine seemed to be uncompetitive with respect to acyl-CoA regardless of the molecular species of the acyl-donor, although susceptibility to the inhibition showed marked dependency on the acyl-CoA species used. Thus, with palmitoyl-CoA as the substrate, the K_i value was approximately twice that obtained with oleoyl-CoA or linoleoyl-CoA. These results indicate that chlorpromazine can bind to the enzyme only after the formation of enzyme–acyl-CoA complex and that the affinity of the enzyme to chlorpromazine produced by the binding of acyl-CoA is higher with unsaturated acyl-CoAs than with saturated species. It is conceivable that chlorpromazine may modify the relative content of each molecular species of phosphatidic acid.

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