Inhibition of palmitoyl CoA deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate

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Abstract Palmitoyl CoA deacylase activity was measured in preparations of rat liver microsomes. Two hypolipidemic agents, chlorophenoxyisobutyrate and betabenzalbutyrate caused inhibition of palmitoyl CoA deacylase in vitro. The I_{50} values for chlorophenoxyisobutyrate and betabenzalbutyrate were 5.0 and 7.5 mM, respectively. The inhibition by both agents was reversible, and both drugs lowered the palmitoyl CoA-binding capacity of microsomes.

The deacylase reaction rate was maximum when the binding of palmitoyl CoA to soluble or microsomal protein was low. Addition of albumin to the reaction mixture resulted in greater binding of palmitoyl CoA to protein and a lower reaction rate. Inhibition of the deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate also was greater when palmitoyl CoA was not protein bound. The inhibition of palmitoyl CoA deacylase by these hypolipidemic agents may contribute to their effects on lipid metabolism.

Supplementary key words binding · albumin · microsomes

PREVIOUS STUDIES have shown that chlorophenoxyisobutyrate and a related compound, betabenzalbutyrate, inhibit the esterification of *sn*-glycerol-3-phosphate by rat liver microsomal preparations (1, 2). No other reactions in the pathway of triglyceride biosynthesis from *sn*-glycerol-3-phosphate are inhibited by either agent. Chlorophenoxyisobutyrate also inhibits acetyl CoA carboxylase activity (3–5) and mevalonate synthesis (6, 7). The inhibition of these key reactions in lipid synthesis may contribute to the lipid-lowering effect of chlorophenoxyisobutyrate. This study reports the effects of these agents on another enzyme of potential importance in lipid synthesis, palmitoyl CoA deacylase. This microsomal enzyme also is inhibited by addition of chlorophenoxyisobutyrate and betabenzalbutyrate in vitro.

Abbreviations: TLC, thin-layer chromatography.

METHODS

Materials

Sodium salts of chlorophenoxyisobutyrate and betabenzalbutyrate were kindly supplied by Ayerst Laboratories and Instituto Biochimico Italiana (IBI), respectively. Fatty acid-poor albumin (fraction V) was purchased from Pentex, Inc. Labeled [1-^{14}C]palmitoyl CoA was purchased from New England Nuclear, and the palmitic acid used as a TLC standard was obtained from Applied Science Laboratories.

Palmitoyl CoA was prepared from palmitoyl chloride (Hormel Institute) as described by Seubert (8). Both labeled and nonlabeled palmitoyl CoA were found to be greater than 95% pure by techniques described previously (9).

Rat liver microsomes were prepared by previous methods (9, 10) with two modifications. The buffer used for homogenization of the liver contained 0.225 M sucrose and 0.05 M Tris–HCl, pH 7.5. After sedimentation, the microsomes were resuspended in the same sucrose–Tris buffer, and aliquots were quickly frozen in dry ice and acetone. Frozen microsomes were thawed once and used for assays. There was no significant change in deacylase activity when microsomes were stored for 2 wk at −15°C.

Products of the incubation system were separated by TLC on 0.25-mm-thick layers of silica gel G developed in hexane–ether–acetic acid 73:25:2 (11). The lipids were identified by comparison with known standards.

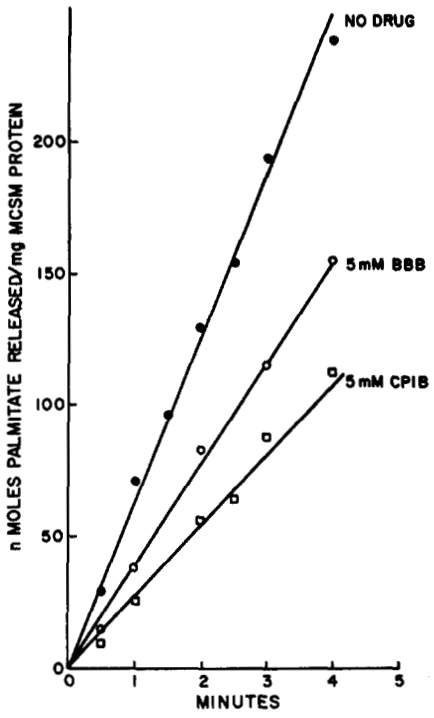

FIG. 1. Palmitoyl CoA deacylase as measured by the standard incubation system. The reaction is linear through 4 min in either the presence or the absence of 5.0 mM chlorophenoxyisobutyrate (CPIB) or betabenzalbutyrate (BBB). Microsomal protein concentration was 0.05 mg/ml.

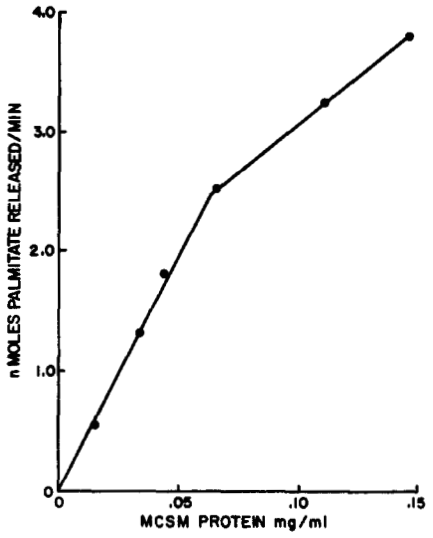

FIG. 2. Effect of increasing microsomal protein on palmitoyl CoA deacylase under the usual incubation conditions. The microsomal (MCSM) protein concentrations represent the final protein concentration in the standard assay volume of 0.8 ml. The reaction was linear with respect to protein through 0.065 mg/ml.

Under the standard incubation conditions, about 75% of the radioactivity chromatographed with palmitate (R_F = 0.48), 20% with polar lipids (R_F = 0), and 5% with diglyceride (R_F = 0.30). These amounts of products are comparable to those reported by Jezyk and Hughes (12).

Binding of palmitoyl CoA to albumin and microsomes was determined by ultrafiltration, sedimentation, and heat precipitation techniques described elsewhere (1). The term free palmitoyl CoA is used in the following discussion to refer to palmitoyl CoA that is not bound to protein or subcellular particles. This would include palmitoyl CoA in the micellar phase and in free solution.

Palmitoyl CoA deacylase

The standard incubation mixture contained 50 μM palmitoyl CoA, 0.01 μCi of [1-^{14}C]palmitoyl CoA, 72.5 mM Tris–HCl, pH 7.5, 2.5 mM dithiothreitol, and 0.04–0.052 mg of microsomal protein in a total volume of 0.80 ml. Reactions were initiated by the addition of microsomes and stopped after a 2-min incubation at 37°C by the addition of 3 ml of chloroform–methanol 1:2. Lipids were extracted by a slight modification of the Bligh and Dyer method (13). Activity was expressed as nanomoles of palmitic acid released per milligram of microsomal protein.

RESULTS

The time course of the palmitoyl CoA deacylase reaction is shown in Fig. 1. Reaction rates were linear through 4 min in the absence or presence of 5 mM chlorophenoxyisobutyrate or betabenzalbutyrate. Both drugs reduced the reaction rate but did not affect the linearity of the reaction with respect to time.

Fig. 2 demonstrates that the reaction rate was proportional to microsomal protein in the range of 0.02 to 0.065 mg/ml. At higher levels the rate became nonlinear. This change at higher protein concentrations was not due to the complete utilization of substrate.

The effect of palmitoyl CoA concentration on reaction rate in the absence and presence of 1.0, 5.0, and 10.0 mM chlorophenoxyisobutyrate and betabenzalbutyrate is shown in Fig. 3. In the absence of drug, the optimum palmitoyl CoA level was 50 μM. However, this changed to approximately 75 μM in the presence of either betabenzalbutyrate or chlorophenoxyisobutyrate. Kinetic interpretations of these results are difficult because of the solubility characteristics of palmitoyl CoA (14, 15). The palmitoyl CoA is present in both free and bound forms under these experimental conditions.

Fig. 4 shows the effect of increasing drug concentrations on the palmitoyl CoA deacylase reaction rate. Both drugs resulted in a similar inhibition pattern. Approximately 50% inhibition was produced by chlorophenoxyisobutyrate and betabenzalbutyrate at 5.0 mM and 7.5 mM, respectively. There was a 10–20% inhibition of palmitoyl CoA deacylase at concentrations of chloro-

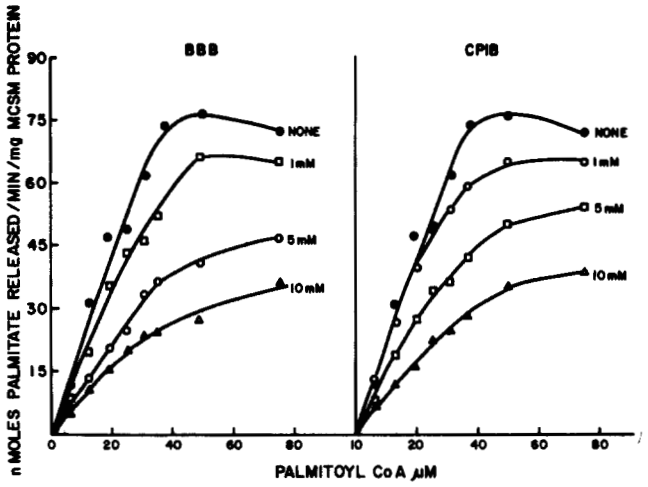

FIG. 3. Palmitoyl CoA deacylase reaction rates with respect to various palmitoyl CoA concentrations in the absence or presence of 1.0, 5.0, and 10.0 mM chlorophenoxyisobutyrate (CPIB) and betabenzalbutyrate (BBB). Both agents were inhibitory at all palmitoyl CoA concentrations. Optimal palmitoyl CoA levels in the absence of drug were 40–50 μM. The usual incubation conditions were used except for the various acyl CoA levels and the addition of drugs. The microsomal protein concentration was 0.065 mg/ml.

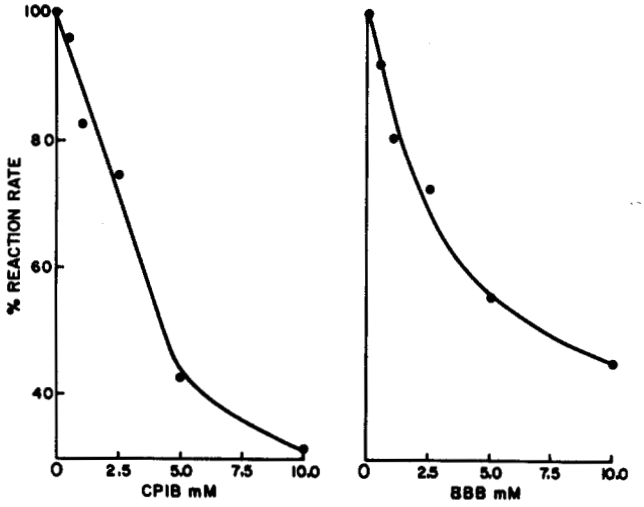

FIG. 4. Effect of increasing concentrations of chlorophenoxyisobutyrate (CPIB) and betabenzalbutyrate (BBB) on palmitoyl CoA deacylase. The usual incubation conditions were followed except for the addition of drugs. The microsomal protein concentration was 0.05 mg/ml.

phenoxyisobutyrate and betabenzalbutyrate comparable to those reported in the serum of rats or humans given these drugs (16).

Table 1 shows the effect of increasing albumin, microsome, and palmitoyl CoA concentrations on the deacylase reaction rate and the distribution of palmitoyl CoA. Experiment A demonstrates that as concentrations of microsomal protein increased there was a reduction of concentration of free palmitoyl CoA and a concomitant increase of bound palmitoyl CoA. There was also a decreased deacylase reaction rate beyond 0.065 mg/ml microsomal protein. This was not due to an increase in microsomal-bound palmitoyl CoA because the addition of albumin, which lowered microsomal-bound palmitoyl CoA (experiment B), did not significantly stimulate the reaction rate. The addition of 0.16 mg/ml albumin lowered microsomal-bound palmitoyl CoA approximately 50% but did not alter free palmitoyl CoA levels or palmitoyl CoA deacylase activity. However, albumin concentrations beyond 0.16 mg/ml lowered both free palmitoyl CoA and deacylase reaction rates. Experiment C shows that as the concentration of palmitoyl CoA increased there was an increased palmitoyl CoA deacylase activity up to 38–50 μM total or 17–26 μM free palmitoyl CoA. The results of these experiments suggest that the deacylase reaction rate correlates with changes in free palmitoyl CoA rather than protein-bound or total palmitoyl CoA concentrations (experiment A). Fig. 5 represents the deacylase reaction rate with respect to free palmitoyl CoA concentrations under the conditions described in Table 1. These results show that 25–30 μM free palmitoyl CoA was optimal in each of these experiments. However, the data also show that approximately 50% of the maximum activity was obtained with 20, 6, and 9 μM free palmitoyl CoA in experiments A, B, and C, respectively. The higher substrate requirement for 50% activity in experiment A, in which microsomal content was varied, could indicate the presence of an inhibitor associated with the microsomes. Therefore, the optimal palmitoyl CoA substrate concentrations for 0.07 and 0.26 mg/ml microsomal protein was determined. Under these conditions, concentrations of approximately 40–50 and 180–200 μM total palmitoyl CoA and 25–30 and 110–120 μM free palmitoyl CoA were optimal for 0.07 and 0.26 mg/ml microsomal protein, respectively. These results and those in Table 2 indicate that the optimal free palmitoyl CoA level increases as microsomal protein rises, further suggesting some form of competitive inhibition by microsomes. Therefore, the optimal substrate concentration is directly dependent on microsomal concentration. This may account for the lack of linearity of the reaction with respect to microsomal protein concentrations above 0.065 mg/ml (Fig. 2).

The inhibition of palmitoyl CoA deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate was studied under various incubation conditions to evaluate possible mechanisms of action. The effects of 5 mM chlorophenoxyisobutyrate on the deacylase reaction rate and on the microsomal binding of palmitoyl CoA were determined at several different concentrations of microsomes, as shown in Table 3, experiment A. The concen-

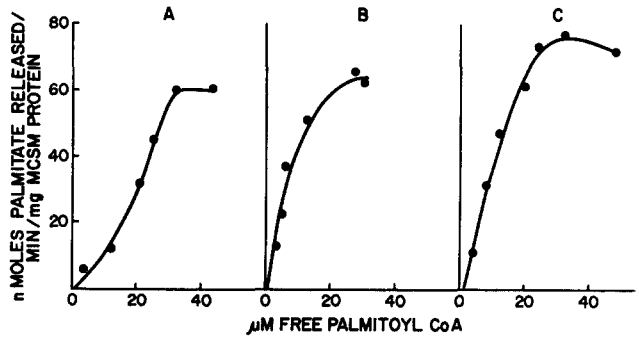

FIG. 5. Palmitoyl CoA deacylase activity was measured in the standard incubation system. The amount of palmitoyl CoA free in solution was measured by techniques previously outlined (1). Free palmitoyl CoA concentrations were varied by several procedures. In experiment A, deacylase activity was measured in the presence of 50 μM palmitoyl CoA at various microsomal protein concentrations (0.033–1.30 mg/ml) in the absence of albumin. Experiment B represents deacylase activity in the presence of 50 μM palmitoyl CoA and 0.065 mg/ml microsomal protein at various albumin concentrations (0–1.25 mg/ml). Experiment C shows deacylase activity in the presence of 0.065 mg/ml microsomal protein without albumin at various concentrations of palmitoyl CoA (10–72 μM). In each case the optimal free palmitoyl CoA concentration was 25–30 μM.

tration of free palmitoyl CoA in the reaction mixture was reduced as increasing amounts of microsomes were added as noted in Table 1. Chlorophenoxyisobutyrate did not alter the binding of palmitoyl CoA by microsomes when added simultaneously with substrate. However, the percentage inhibition by the drug was reduced as the microsomal protein concentration was increased. Both the reduced reaction rate and the decreased inhibition by chlorophenoxyisobutyrate were prevented by the addition of excess palmitoyl CoA, as noted in Table 3, experiment B. These results would suggest that the inhibitions by chlorophenoxyisobutyrate and high microsomal concentrations are not entirely additive and that the maximal effect of the drug is noted only when microsomal inhibition is overcome by high substrate concentrations. However, in contrast to the effect of increasing microsomal concentration, the inhibition by chlorophenoxyisobutyrate does not seem competitive in relation to either microsomal-bound or free palmitoyl CoA (Table 3). High concentrations of substrate result in a greater proportional inhibition by this drug.

TABLE 1. Effect of increasing concentrations of microsomes, albumin, and palmitoyl CoA on the distribution of palmitoyl CoA and on palmitoyl CoA deacylase activity[a]

Experiment	Protein Added		Palmitoyl CoA	Distribution of Palmitoyl CoA[b]			Relative Reaction Rate[c]
	MCSM	Albumin		MCSM Bound	Albumin Bound	Free	
	mg/ml	*mg/ml*	*μM*		*nmoles*		
A	0.015		50	3.2		36.8	1.08
	0.030		50	6.0		34.0	1.00
	0.065		50	14.4		25.6	1.00
	0.130		50	20.2		19.8	0.75
	0.210		50	23.0		17.0	0.53
	0.650		50	30.2		9.8	0.20
	1.300		50	36.0		4.0	0.10
B	0.065		50	16.3		23.7	1.00
	0.065	0.16	50	7.9	9.7	22.4	1.10
	0.065	0.31	50	5.8	24.3	9.9	0.85
	0.065	0.62	50	1.6	33.8	4.6	0.62
	0.065	0.94	50	0.3	36.2	3.6	0.38
	0.065	1.25	50	0.4	37.2	2 4	0.22
C	0.070		6.25	2.3		2.7	0.15
	0.070		12.50	4.3		5.7	0.45
	0.070		18.75	6.5		8.5	0.61
	0.070		25.00	9.0		11.0	0.64
	0.070		31.25	10.8		14.2	0.80
	0.070		37.50	12.9		17.1	0.96
	0.070		50.00	14.0		26.0	1.00
	0.070		75.00	16.8		43.2	0.94

[a] Deacylase activity was measured in the presence of increasing microsome (MCSM) (A), albumin (B), and palmitoyl CoA (C) concentrations as indicated.

[b] Palmitoyl CoA binding was measured by techniques described previously (1). At the highest concentration of microsomal protein, less than 40% of the palmitoyl CoA had been utilized.

[c] Reaction rate was expressed in relation to the activity when 0.065–0.070 mg of microsomal protein/ml was used in the absence of albumin. Therefore, when release of palmitate was directly proportional to change in microsomal protein concentration, the relative reaction rate was 1.0.

Chlorophenoxyisobutyrate may also reduce microsomal binding of palmitoyl CoA when incubated with microsomes prior to substrate addition (Table 4, expt. A). Under these incubation conditions the drug had an additional inhibitory effect on the deacylase (expt. B, 2 and 4) at both high and low microsomal protein concentrations.

These inhibitions of deacylase activity do not represent irreversible denaturation of the enzyme, as shown in Table 4C. Incubation of microsomes with chlorophenoxyisobutyrate followed by sedimentation and washing to remove the drug restores full activity.

DISCUSSION

Chlorophenoxyisobutyrate has a variety of effects on mammalian lipid metabolism. These include reduced adipose tissue lipolysis (17), inhibition of acetyl CoA carboxylase (3–5), decreased hepatic triglyceride release (18–20), decreased hepatic glycerolipid formation (21), decreased lipid synthesis in skin tissue (22), increased peripheral uptake of serum triglyceride (23), reduced cholesterol biosynthesis (6, 7), reduced adenyl cyclase activity (24), increase in adipose tissue lipoprotein lipase (25), and displacement of thyroxine from serum into liver (26). Several of these effects in combination may contribute to the reduction of serum lipids caused by this agent.

The present study shows that liver palmitoyl CoA deacylase is inhibited by chlorophenoxyisobutyrate and the related compound betabenzalbutyrate. Both drugs are reversible inhibitors and inhibit the reaction under a wide variety of substrate and microsome concentrations.

The reaction rate for palmitoyl CoA deacylase appears to be greatest when the free palmitoyl CoA concentration is optimal. Reducing free palmitoyl CoA without change in total palmitoyl CoA by binding to microsomes or albumin reduces the reaction rate. However, maintaining relatively constant free palmitoyl CoA levels does not overcome the reduced activity produced by increasing microsome concentrations. Only when the free palmitoyl concentration is increased proportionately with respect to the microsomal level does the deacylase activity return to normal levels. This suggests that some competitive inhibitor with respect to free palmitoyl CoA levels is associated with the microsomes.

The inhibition of palmitoyl CoA deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate is apparently greatest when unbound palmitoyl CoA is the major substrate. For example, 5 mM chlorophenoxyisobutyrate caused 48% inhibition under the standard incubation conditions in which 60% of the palmitoyl CoA was unbound. The inhibition was only 20% when

TABLE 2. Effect of increasing concentrations of microsomes and palmitoyl CoA on palmitoyl CoA deacylase activity[a]

Experiment	MCSM	Palmitoyl CoA	nmoles Palmitoyl CoA[b] MCSM Bound	Free	Reaction[c] Rate
	mg/ml	*μM*			
A	0.070	40	12.2	19.8	57.3
	0.140	80	25.6	38.4	63.1
	0.340	190	60.8	91.2	57.0
B	0.060	40	12.9	17.1	58.9
	0.120	55	21.0	24.0	50.2
	0.240	75	28.0	32.0	38.4
	0.360	115	42.0	48.0	37.0

[a] Palmitoyl CoA deacylase activity was measured in the presence of increasing microsomal (MCSM) and palmitoyl CoA concentrations as indicated. The assay mixtures were incubated for 2 min, and less than 40% of the palmitoyl CoA was utilized at the highest protein concentrations.

[b] Binding of palmitoyl CoA was measured as described previously (1).

[c] Reaction rate is expressed as nmoles of palmitate released/min/mg MCSM protein.

TABLE 3. Inhibition of palmitoyl CoA deacylase by 5 mM chlorophenoxyisobutyrate at various concentrations of microsomes and palmitoyl CoA[a]

Experiment	MCSM	Palmitoyl CoA	Free Palmitoyl CoA[b]	CPIB,[c] 5 mM	Reaction[d] Rate	Inhibition
	mg/ml	*μM*	*nmoles*			%
A	0.065	50		−	50.4	
			25.6			47.8
	0.065	50		+	26.3	
	0.130	50		−	37.7	
			19.8			35.5
	0.130	50		+	24.3	
	0.210	50		−	26.9	
			17.0			36.0
	0.210	50		+	17.2	
	0.650	50		−	9.9	
			9.8			24.2
	0.650	50		+	7.5	
	1.300	50		−	5.1	
			4.0			29.5
	1.300	50		+	3.6	
B	0.070	40		−	57.3	
			19.8			42.2
	0.070	40		+	33.1	
	0.140	80		−	63.1	
			38.4			42.6
	0.140	80		+	36.2	
	0.340	190		−	57.0	
			91.2			41.6
	0.340	190		+	33.3	

[a] The inhibition of palmitoyl CoA deacylase by 5 mM CPIB was studied in the presence of increasing microsome (MCSM) concentrations at 50 μM palmitoyl CoA (experiment A) and increasing concentrations of both microsomes and palmitoyl CoA (experiment B).

[b] The amount of "free" (unbound) palmitoyl CoA was estimated by techniques described previously (1).

[c] Tris–HCl (0.05 M) buffer, pH 7.5, was added with the appropriate amount of drug.

[d] Reaction rate was expressed as nmoles of palmitate released/min/mg MCSM protein.

TABLE 4. Effect of chlorophenoxyisobutyrate on microsomal palmitoyl CoA deacylase and binding[a]

Experiment	MCSM	Palmitoyl CoA	Palmitoyl CoA Bound[b]	CPIB, 5 mM	Reaction Rate[h]	Inhibition
	mg/ml	*μM*	*nmoles*			*%*
A						
1	0.10	50	19.0	−		
2[c]	0.10	50	18.8	+		
3	0.10	50	18.0	+		
4[d]	0.10	50	4.3	+		
B						
1[e]	0.07	50		−	46.8	
						56.0
	0.07	50		+	20.7	
2[f]	0.07	50		−	48.4	
						73.6
	0.07	50		+	12.8	
3[e]	0.14	50		−	31.3	
						43.5
	0.14	50		+	17.7	
4[f]	0.14	50		−	30.9	
						69.6
	0.14	50		+	9.4	
C[g]	0.07	50		+	53.9	
	0.07	50		−	55.5	
	0.20	100		+	54.1	
	0.20	100		−	52.2	

[a] Microsomal palmitoyl CoA deacylase activity and binding was measured under the indicated conditions. Each control (minus drug) assay was treated exactly the same as the drug assay with the exception of adding 0.05 M Tris–HCl buffer, pH 7.5, in place of the drug.

[b] Palmitoyl CoA binding by heat inactivated microsomes was determined by techniques described previously (1).

[c] Experiments A-1 and A-2 represent the microsomal binding of palmitoyl CoA when all assay components were mixed together without and with chlorophenoxyisobutyrate (5 mM) before the addition of microsomes.

[d] Experiments A-3 and A-4 show the amount of palmitoyl CoA bound when microsomes and palmitoyl CoA were mixed before the addition of chlorophenoxyisobutyrate and when chlorophenoxyisobutyrate and microsomes were mixed before the addition of palmitoyl CoA, respectively.

[e] Experiments B-1 and B-3 represent the inhibition of palmitoyl CoA deacylase by chlorophenoxyisobutyrate under the usual incubation conditions in which the reaction was started by the addition of microsomes.

[f] Experiments B-2 and B-4 show the incubation of chlorophenoxyisobutyrate when it was preincubated with the microsomes prior to the addition of palmitoyl CoA.

[g] Experiment C shows the deacylase activity of microsomes that were sedimented from a mixture containing buffer or buffer with 5 mM chlorophenoxyisobutyrate, washed, and resuspended in buffer containing no drug.

[h] Reaction rate was expressed as nmoles of palmitate released/min/mg MCSM protein.

90% of the palmitoyl CoA was microsomal bound. When additional palmitoyl CoA was added under these latter conditions, the percentage inhibition by chlorophenoxyisobutyrate was restored to the higher level. It seems likely that chlorophenoxyisobutyrate is a direct inhibitor of microsomal palmitoyl CoA deacylase and this inhibition is not strictly competitive. Furthermore, the agent will inhibit microsomal binding of palmitoyl CoA when added prior to the substrate, further reducing deacylase activity. The precise mechanism of inhibition cannot be determined in a complex mixture containing particulate enzyme and protein-bound substrates.

The importance of palmitoyl CoA deacylase in regulating glycerolipid metabolism is not clear. The initial reaction in this pathway, *sn*-glycerol-3-phosphate acyltransferase may be affected by the level of microsomal-bound palmitoyl CoA (1). At levels of bound palmitoyl CoA that exceed 20 nmoles/mg microsomal protein, *sn*-glycerol-3-phosphate acyltransferase activity is reduced (1). Also, the activity of acetyl CoA carboxylase may be influenced by acyl CoA levels (27, 28). Therefore, inhibition of deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate might indirectly reduce lipid formation by increasing the cellular concentration of palmitoyl CoA to inhibitory concentrations.

There is no present information about the intracellular distribution of acyl CoA derivatives in liver. Tubbs and Garland (29) reported a total acyl CoA concentration of 40–160 nmoles/g rat liver depending on diet. The acyl CoA levels used in the present studies are comparable to their (29) in vivo acyl CoA concentrations. The albumin concentration in liver has been estimated at 0.5 mg/g (30). This amount of albumin would bind about 30 nmoles of palmitoyl CoA in vitro. The remaining palmitoyl CoA in vivo is probably bound to other intracellular proteins, including microsomal proteins. Therefore, the inhibition of acyl CoA deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate in relation to the bound palmitoyl CoA substrate may be of greater significance in the intact animal. Interpretation of the physiological importance of this inhibition requires further information on the intracellular distribution and concentration of long-chain fatty acyl CoA and the hypolipidemic agents.

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