The Effect of 2,4,6,8-Tetramethyldecanoic Acid on the Hydrolysis and Acylation of Phospholipid by Lipase in Isooctane

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The effect of an unusual natural fatty acid, 2,4,6,8-tetramethyldecanoic acid (TMDA), on the hydrolysis and acylation of phospholipid by lipase in isooctane was studied. Lipases examined were from *Rhizopus delemar, Candida cylindracea* and *Pseudomonas* sp. The lipase was dispersed in isooctane by dioleoylphosphatidylethanolamine (DOPE) with or without the fatty acid and decanoyl-lysophosphatidylcholine (LPC). The effect of TMDA on both the hydrolysis of DOPE and the acylation of LPC was compared with that of decanoic acid (DA) by varying the water content. At the higher water content, the hydrolysis of DOPE was enhanced or trace amount of phosphatidylcholine was produced. Hydrolysis was enhanced more by the addition of TMDA than by DA. The effect of TMDA on the acylation of LPC was similar to that of DA.

KEY WORDS: Acylation of lysophosphatidylcholine, decanoic acid, dioleoylphosphatidylethanolamine, hydrolysis of phosphatidylethanolamine, lipase, reverse micelles, 2,4,6,8-tetramethyldecanoic acid.

There are many varieties of fatty acids in nature. Among them, only six varieties account for more than 95% of the fatty acids of membrane constituents (1). Large numbers of naturally occurring unusual fatty acids exist in small quantities or in peculiar natural sources (1). Their biological functions are at present little known. 2,4,6,8-Tetramethyldecanoic acid (TMDA) was found in the preen gland of the goose (*Anser domesticus*) as a wax ester (2,3). TMDA is a multi-branched fatty acid and the major (95%) fatty acid component of that preen gland. Neither biological nor physicochemical properties of TMDA have been determined precisely.

Lipase is an enzyme that hydrolyzes esters, such as glycerides, waxes and sugar esters and reversely catalyzes the esterification reaction. It is also known that lipases hydrolyze phosphatidylcholines (PC), such as phospholipase A_1 (4,5), and catalyze the acidolysis of PC (6). Lipases are active in organic solvent, *e.g.*, activity of lipase in water-in-oil microemulsion or reverse micelles (7,8) was reported. To disperse lipase in organic solvent with phospholipid, a cosurfactant, such as palmitic acid or hexanol, has been commonly used (9). The effect of the type of fatty acid on the hydrolysis and acylation of phospholipid by lipase in such a dispersion has not yet been precisely determined.

In this paper we describe the effect of TMDA on the hydrolysis and acylation of phospholipid by lipase in isooctane, and show for the first time the property of TMDA in relation to phospholipid reverse micelles. Addition of TMDA apparently enhanced the hydrolysis of dioleoylphosphatidylethanolamine (DOPE) compared with the additon of decanoic acid (DA), while TMDA and DA affected the acylation of lysophosphatidylcholine (LPC) to the same extent.

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EXPERIMENTAL PROCEDURES

Lipase from *Rhizopus delemar* was supplied by Fluka (Neu-Elm, Germany). Lipases from *Candida cylindracea* and *Pseudomonas* sp. were obtained from Boehringer Mannheim (Mannheim, Germany). Organic solvents were of analytical grade by Merck (Darmstadt, Germany). The TMDA was kindly supplied by Dr. M. Morr from Gesellschaft für Biotechnologische Forschung (Germany). Phospholipids, N-tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and other chemicals were purchased from Sigma (St. Louis, MO). TLC plates (silica gel plate with concentrating zone, No. 11845) were purchased from Merck.

Each lipase was prepared as a buffer solution (0.05 M of TES-NaOH, pH 7). The concentrations of the lipase solutions were calculated from the triacylglycerol hydrolytic activity at pH 8. For hydrolysis and acylation, lipase solutions of 750 and 3,000 U/mL, and of 3,000 and 30,000 U/mL were prepared, respectively.

The hydrolysis of DOPE by lipase in isooctane was determined as follows: The buffer solution or the lipase (from *Rhizopus delemar*) solution was added to the isooctane solution of DOPE (100 mM) with or without fatty acid (100 mM) at a w_0 value (the water/DOPE molar ratio) of either 1.7 or 7. After one day of incubation at 37°C, the mixture was analyzed by thin-layer chromatography (TLC). The fractions of DOPE and lysophosphatidylethanolamine (LPE) were scraped off, extracted with chloroform-methanol (1:2, vol/vol) and analyzed for phosphorus according to the method of Bartlett (10).

The PC synthetic activity of lipase in isooctane was examined as follows: The mixture of DOPE, decanoyl-LPC and fatty acid (100 mM:50 mM:100 mM) in isooctane was added to the enzyme solution at the w_0 value of either 0.7 or 7. After one day of incubation at 37°C, the products were separated by TLC and identified by their R_f values and by staining with reagents such as molybdenum blue, ninhydrin and Dragendorff reagent. PC production was determined by its phosphorus content (10).

RESULTS AND DISCUSSION

Table 1 shows the effects of TMDA and the w_0 value on the DOPE hydrolytic activity of lipase. The molar ratio of LPE or DOPE to the initial DOPE was expressed as a relative value to the control's average value, *i.e.*, $(LPE/PE_0)/(LPE/PE_0)_{Cont}$ or $(PE/PE_0)/(PE/PE_0)_{Cont}$.

At $w_0 = 1.7$, the hydrolysis of DOPE was not affected by the addition of either TMDA or DA. However, the hydrolysis of DOPE was enhanced by raising the w_0 value (see [g] in Table 1). The difference was most pronounced when the lipase concentration was raised from 1.5 U to 6 U. By the analogy of PC, it is estimated that the fatty acid works as a cosurfactant, that the w_0 value can be raised, and that the higher w_0 value is suitable for the lipase activity (9).

The effect of TMDA was larger than that of DA, as shown in Table 1 (see [e]). Although both TMDA and DA

TABLE 1

Hydrolysis of DOPE by Lipase in Isooctane After One-Day Incubation at 37°C

Sample composition[a]	$(LPE/PE_0)/(LPE/PE_0)_{Cont}$[b]	$(PE/PE_0)/(PE/PE_0)_{Cont}$[b]
Control = DOPE/B[c] (w_0 = 1.7)[d]	1.00 ± 0.26 (n = 3)	1.00 ± 0.03 (n = 5)
DOPE/DA/B (w_0 = 1.7)	1.01 ± 0.32 (n = 7)	1.11 ± 0.34 (n = 3)
DOPE/DA/B[e] (w_0 = 7)	1.00 ± 0.52 (n = 4)	0.98 ± 0.19 (n = 4)
DOPE/TMDA/B[e] (w_0 = 7)	1.07 ± 0.43 (n = 4)	1.05 ± 0.10 (n = 3)
DOPE/DA/L[f] (1.5 U, w_0 = 1.7)	1.12 ± 0.19 (n = 7)	0.95 ± 0.43 (n = 3)
DOPE/DA/L (1.5 U, w_0 = 7)	1.48 ± 0.28 (n = 4)	0.38 ± 0.03 (n = 4)
DOPE/DA/L[e] (6 U, w_0 = 7)	4.02 ± 0.86 (n = 4)	0.35 ± 0.09 (n = 4)
DOPE/TMDA/L[g] (1.5 U, w_0 = 1.7)	1.06 ± 0.48 (n = 6)	0.80 ± 0.20 (n = 3)
DOPE/TMDA/L[g] (1.5 U, w_0 = 7)	2.23 ± 0.56 (n = 4)	0.32 ± 0.03 (n = 4)
DOPE/TMDA/L[e] (6 U, w_0 = 7)	7.17 ± 0.91 (n = 4)	0.14 ± 0.05 (n = 3)

[a]Concentrations of DOPE and DA/TMDA were 100 mM and 100 mM, respectively.

[b]Molar ratio of LPE or DOPE to the initial DOPE, expressed as the relative value to the control's average value. Results were expressed as the average ± standard deviation. Number of repetition was expressed as n.

[c]Buffer solution (0.05 M of TES-NaOH, pH 7).

[d]Initial molar ratio of water to DOPE.

[e]Hydrolysis was caused by lipase at the 1% significance level. Addition of TMDA enhanced hydrolysis stronger than DA ($P < 0.02$).

[f]Buffer solution of lipase from *Rhizopus delemar*. The unit values for triacylglycerol hydrolysis at pH 8 were also described in parenthesis.

[g]Hydrolysis was enhanced at the larger w_0 value ($P < 0.02$).

TABLE 2

R_f Values of the PC Produced After One-Day Incubation at 37°C[a]

	Solvent A[b]	Solvent B[c]	Solvent C[d]
Standard PE	0.56	0.62	0.67
Standard LPE	0.22	0.34	0.25
Standard PC	0.28	0.27	0.16
Produced PC	0.28	0.28	0.18
Standard LPC	0.09	0.08	0.04

[a]The produced PC was concentrated and stained with molybdenum blue and Dragendorff reagent.

[b]Mixture of chloroform/methanol/25% ammonium hydroxide (65:25:5, vol/vol).

[c]Mixture of chloroform/methanol/water (65:25:4, vol/vol).

[d]Mixture of chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, vol/vol).

TABLE 3

Yield of PC from LPC After One-Day Incubation at 37°C at pH 7

Sample composition[a]	PC/LPC_0[b]
DOPE/LPC/TMDA/L[c] (*R. delemar*) (w_0 = 7)[d]	0.0041 ± 0.0008 (n = 4)
DOPE/LPC/DA/L (*R. delemar*) (w_0 = 7)	0.0047 ± 0.0002 (n = 3)
DOPE/LPC/DA/L (*C. cylindracea*) (w_0 = 7)	0.0035 ± 0.0016 (n = 4)
DOPE/LPC/DA/L (*C. cylindracea*) (w_0 = 0.7)	n.d.[e]
DOPE/LPC/DA/L (*P.* sp.) (w_0 = 7)	0.0056 ± 0.0009 (n = 5)

[a]Concentrations of DOPE, LPC and DA/TMDA were 100 mM, 50 mM and 100 mM, respectively.

[b]Molar ratio of the produced PC to the initial LPC. Number of repetitions was expressed as n. Differences are not significant at the 1% level.

[c]Buffer solution of lipase (6 U for triacylglycerol hydrolysis at pH 8).

[d]Initial molar ratio of water to DOPE.

[e]Not determined as no PC was observed.

have the same chainlength, TMDA is more bulky than DA due to the four methyl groups. It is hard to imagine that the branched fatty acid directly activates lipase. Rather, similar to the effect of diacylglycerol on the phospholipase A_2 activity (11), it is expected that the four methyl groups of TMDA disorder the DOPE aggregation in isooctane. Rakhimov *et al.* (12) also showed the indirect effect of Triton X-100 on the phospholipase A_2 activity in the reverse micelles of Triton X-100 PC in benzene.

The produced PC was identified by its R_f value and by its staining reaction. Table 2 shows R_f values of the produced PC and standard phospholipids in the three different developing solvent systems. This PC fraction had the same R_f value as that of the standard PC in every (basic, neutral or acidic) solvent system, and gave positive reaction to molybdenum blue and Dragendorff reagent.

Table 3 shows the effect of TMDA and the w_0 value on the yield of PC from LPC. The yield of PC was as low as the reported value (13) of phospholipase A_2-catalyzed PC production in several organic solvents.

The effect of TMDA was the same as that of DA. However, gas chromatography analysis of the fatty acid composition revealed that the produced PC contained no TMDA (data not shown). The mechanism of PC production is still under investigation.

On the other hand, water content was related to the activity of lipase. Contrary to the expectation that the lower water content might enhance the acylation of LPC by lipase, PC production was not observed at $w_0 = 0.7$. The higher w_0 value was required for acylation, as well as for hydrolysis.

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