Regulation of Activities of Cytidine 5′-Diphospho-Choline: 1-*O*-Alkyl-2-Acetyl-*sn*-Glycerol Cholinephosphotransferase, an Enzyme Responsible for *de novo* Synthesis of Platelet-Activating Factor, by Membrane Phospholipids

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Cytidine 5′-diphospho (CDP)-choline: 1-*O*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase (AAG-CPT), an enzyme responsible for *de novo* synthesis of platelet-activating factor (PAF), was solubilized from porcine spleen microsomes using digitonin. Although the activity of the solubilized enzyme was relatively stable, further purification by sequential chromatography on Toyopearl HW-65 gel filtration and diethylaminoethyl (DEAE)-Toyopearl 650 caused a remarkable decrease in enzyme activity, which was partially recovered by the exogenously addition of phospholipids such as egg phosphatidylcholine, dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine, dioleoylphosphatidylglycerol, and dioleoylphosphatidylserine. In contrast, dioleoylphosphatidic acid (DOPA) showed an inhibitory effect on enzyme activity. In addition, lysophospholipids such as monooleoylphosphatidylcholine, monooleoylphosphatidylethanolamine, monooleoylphosphatidylglycerol, and monooleoylphosphatidic acid showed an inhibitory effect on the enzyme activity. When DOPA was concomitantly added with DOPC, the enzyme activity reactivated by DOPC decreased with the ratio of DOPA to DOPC. Furthermore, we examined whether phosphatidic acid (PA) or lysophospholipids had an inhibitory effect on AAG-CPT activity under more physiological conditions. Treatment of microsomes with exogenously added phospholipase D or phospholipase A_2 resulted in a decrease in AAG-CPT. In addition, when endogenous phospholipase D was activated by fatty acids such as oleic acid and arachidonic acid to generate PA in the porcine microsomes, the enzyme activity was significantly inhibited. The molecular weight of the enzyme solubilized from porcine spleen microsomes was estimated to be 440 kDa based on gel-filtration column chromatography on Toyopearl HW-65, suggesting that this enzyme formed a complex with other protein molecules and membrane phospholipids, and that these phospholipids were necessary to maintain the enzyme activity. Our results indicate that environmental membrane phospholipids containing PA and/or lysophospholipids are important factors in the regulation of the enzyme for *de novo* synthesis of PAF.

Key words —— platelet-activating factor, cholinephosphotransferase, phosphatidic acid, lysophosphatidic acid, phospholipid

INTRODUCTION

Platelet-activating factor (PAF) is a phospholipid with a wide range of biological and pharmacological activities, including aggregation and degranulation of platelets and neutrophils, bronchoconstriction, systemic hypotension, anaphylaxis, and inflammatory and allergic responses.[1] The biosynthesis of PAF is known to occur via *de novo* or remodeling pathways. The *de novo* pathway is associated with the production of the basal level of PAF required to maintain the normal physiological function involving regulation of blood pressure, while the remodeling pathway provides PAF relevant to pathological states in response to inflammatory stimuli. The final step in the *de novo* synthesis of PAF is catalyzed by the reaction in which the phosphocholine base group from cytidine 5′-

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diphospho (CDP)-choline is transferred to 1-*O*-alkyl-2-acetyl-*sn*-glycerol.[2] CDP-choline: 1-*O*-alkyl-2-acetyl-*sn*-glycerol cholinephosphotransferase (AAG-CPT), responsible for this reaction, is clearly distinguished from a similar enzyme, CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (DG-DPT), which catalyzes the transfer of phosphocholine to diacylglycerol based on several lines of biochemical evidence. Particularly, AAG-CPT is stimulated by dithiothreitol (DTT), while DG-CPT is inhibited by DTT.[3,4] On the other hand, both enzymes share some similar biochemical properties such as a requirement for Mg^{2+} for enzyme activities.[3,4] AAG-CPT is widely distributed among mammalian tissues[3–5] and is located on the cytoplasmic surface of the endoplasmic reticulum.[4]

To date, this enzyme has been investigated using microsomal fractions of mammalian tissues, because no method for solubilizing the enzyme in an active form from the microsome membrane is known. To understand the regulatory mechanism of this enzyme, we attempted solubilization and further purification of the enzyme from porcine spleen microsomes. Although enzyme activity was solubilized using digitonin in an active form, further purification by sequential column chromatography caused remarkable loss of enzyme activity. Since solubilized enzyme was found to form a complex with other protein molecules and membrane phospholipids upon gel-filtration column chromatography, we examined whether membrane phospholipids could recover the decreased enzyme activity. We found that some diacylphospholipids such as phosphatidylcholine (PC) reactivated the decreased enzyme activity. On the other hand, phosphatidic acid (PA) and lysophospholipids showed inhibitory effects on the enzyme activity. Furthermore, the addition of PA or lysophospholipids with PC to solubilized enzyme inhibited the enzyme activity depending on the ratio of these phospholipids to PC. To determine whether the inhibition of enzyme activity by PA or lysophospholipids occurred in a more nearly physiological system, we examined the enzyme activity in intact microsomes in which PA or lysophospholipids were accumulated by treatment with exogenously added phospholipase D or phospholipase A_2. In addition, we examined the enzyme activity of intact microsomes in which PA was generated by activation of endogenous phospholipase D with fatty acid such as oleic acid.

MATERIALS AND METHODS

Materials —— CDP [methyl-^{14}C] choline was obtained from Amersham Pharmaceutical Biotech (U.K.). 1-*O*-Alkyl-2-acetyl-*sn*-glycerol was obtained from Calbiochem (CA, U.S.A.). Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and dioleoylphosphatidylglycerol (DOPG) were obtained from Sigma (St. Louis, MO, U.S.A.). Dioleoylphosphatidylserine (DOPS), dioleoylphosphatidic acid (DOPA), monooleoylphosphatidylcholine (MOPC), monooleoylethanolamine (MOPE), monooleoylphosphatidylglycerol (MOPG), monooleoylphosphatidylserine (MOPS), and monooleoylphosphatidic acid (MOPA) were obtained from Avanti (AL, U.S.A.). Toyopearl HW-65 and DEAE-Toyopearl 650 were obtained from Tosoh (Japan). *Streptomyces chromofuscus* (*S. chromofuscus*) phospholipase D was purchased from Sigma. *Naja naja* phospholipase A_2 was obtained from Sigma.

Preparation of Microsomes —— Porcine spleens (Tokyo Shibaura Zoki, Japan) were homogenized with 3 volumes of Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M and ethylenediaminetetraacetic acid (EDTA) 1 mM. The homogenates were centrifuged at 150 *g* for 5 min at 4°C. The resulting supernatants were further centrifuged at 1000 *g* for 10 min to remove the nuclei. The supernatant obtained was centrifuged at 20000 *g* for 10 min to remove mitochondria and lysosomes, and then the resulting supernatants were further centrifuged at 105000 *g* for 60 min. The obtained pellet was washed twice with Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M, suspended in the same medium, and used as the microsomal fraction.

Assay of AAG-CPT —— The assay mixture consisted of Tris–HCl 100 mM (pH 8.0), EDTA 0.5 mM, $MgCl_2$ 10 mM, DTT 5 mM, phenylmethylsulfonylfluoride (PMSF) 1 mM, bovine serum albumin (BSA) 1 mg/ml, 1-*O*-alkyl-2-acetyl-*sn*-glycerol 600 μM, CDP [methyl-^{14}C]choline (0.02–0.04 μCi) 100 μM, and enzyme in a total volume of 200 μl. The enzyme reaction was carried out at 37°C for 3–10 min. Radiolabeled PAF was separated from unreacted CDP [methyl-^{14}C] choline by the method of Bligh and Dyer,[6] with a slight modification. The lower chloroform layer was transferred into scintillation vials; radioactivity was determined using a liquid scintillation counter (Aloka).

Solubilization of AAG-CPT and Chromatography —— A microsome pellet was suspended in

Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M and 4% (w/v) digitonin at the final protein concentration of 5 mg/ml. The mixture was sonicated using a microchip until the solution turned clear at maximum intensity. The sonicated mixture was then centrifuged at 100000 *g* for 30 min. The resulting supernatant was used as the solubilized enzyme. Enzyme solubilized with digitonin (6 ml) was applied onto a column of Toyopearl HW-65 (80 ml) previously equilibrated with Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M and 0.2% (w/v) Tween 80. The active fractions were pooled and then applied onto a column of DEAE-Toyopearl 650 (2 ml) previously equilibrated with Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M and 0.2% (w/v) Tween 80. The column was washed with the same solution and the absorbed proteins were eluted step wise by adding the above solution containing NaCl 0.1, 0.2, 0.3, and 0.6 M successively. The fractions of 0.5 ml were collected, and the active fractions eluted with NaCl 0.2 M containing solution were pooled.

Activation of Endogenous Phospholipase D in Porcine Spleen Microsomes —— Porcine spleen microsomes (71 μg, 0.1 μmol phosphorus) were reconstituted in the solution consisting of β,β-dimethylglutarate 25 mM (pH 6.5), $MgCl_2$ 2 mM, and propranolol 0.2 mM in the presence of 0.05–1 mM of fatty acids in a total volume of 100 μl. The mixture was incubated at 37°C for 60 min, and then AAG-CPT activity was determined.

Protein Determination —— Protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce) using BSA as a protein standard. In the column chromatography, protein concentrations were monitored by measuring absorbance at 280 nm.

RESULTS

Solubilization of AAG-CPT Activity from Porcine Spleen Microsomes and Inactivation of AAG-CPT after Column Chromatography

Renooij and Snyder reported that AAG-CPT was mainly located in microsomes and that the spleen exhibited the highest enzyme activity in the rat.[3] Thus, to attempt solubilization and further purification of this enzyme, we used porcine spleen from which a larger amount of enzyme is readily available as the enzyme source. Consistent with their results obtained using rat tissues, porcine spleen microsomes showed resistance of enzyme activity to DTT and a requirement for Mg^{2+} for enzyme activity (data not shown). Of the detergents tested, only a few were effective in solubilizing the enzyme activity. Sodium dodecyl sulfate (SDS) (5 mM), Zwittergent 3–12 (5 mM), dodecyltrimethylammoniumchloride (5 mM), Triton X-100 (1%), and $C_{12}E_8$ (1%) abolished enzyme activity completely. Although Tween 80 (1%) did not inhibit enzyme activity, this detergent showed no ability to solubilize the enzyme activity. 3-[(3-Cholamidopropyl) dimethyl ammonio] propanesulfonic acid (CHAPS) (5 mM), dodecylmaltodie (5 mM), and digitonin (1%) had the ability to solubilize the enzyme activity. We further examined the effect of detergent/protein ratios on the solubilizing efficiency of enzyme activity. No enzyme activity was solubilized by digitonin up to the ratio (w/w) of 0.35, but maximum solubilization was achieved using digitonin at the ratio (w/w) of around 8.0. Thus we determined protein and digitonin concentrations as 5 mg/ml and 4%(w/v), respectively, so that the digitonin/protein ratio (w/w) was 8.0. Furthermore, we attempted to purify this enzyme by column chromatography. In gel-filtration column chromatography on Toyopearl HW-65, enzyme activity was eluted as a single peak away from the void volume, and the molecular weight of the enzyme was estimated to be 400 kDa (Fig. 1). Pooled fractions with enzyme activity were further separated using ion-exchange column chromatography on DEAE-Toyopearl 650 (data not shown). Enzyme activity was adsorbed to the column and eluted by NaCl 0.2 M containing solution. No other enzyme activity emerged using NaCl up to 0.6 M. However, recovery of total enzyme activity was only 10% of solubilized enzyme activity and enzyme-specific activity was decreased after purification by sequential column chromatography, as compared with the original specific activity.

Effects of Exogenously Added Diacylphospholipids on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

We examined whether the phospholipids had the ability to recover the enzyme activity that decreased during the process of column chromatography. As shown in Fig. 2, the enzyme activity was reactivated by exogenously added egg PC in a dose–dependent manner. Maximum enzyme activity was shown at egg PC/protein ratios (w/w) of more than 1.25. Furthermore, we examined the effects of various types of membrane phospholipids on enzyme activity ob-

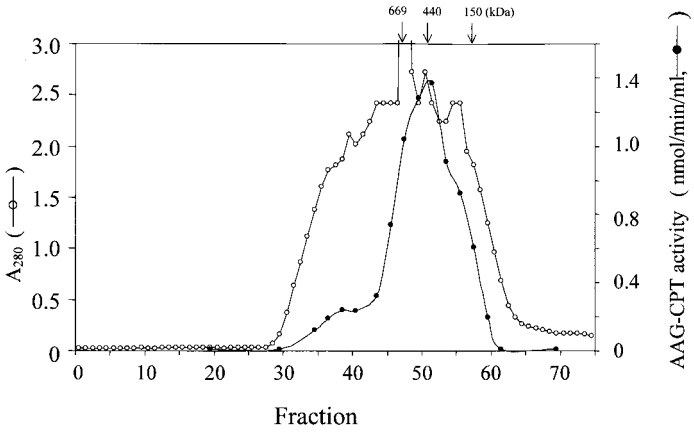

Fig. 1. Column Chromatography of Solubilized Enzyme on Toyopearl HW-65 (A)

Solubilized enzyme from porcine microsomes was applied on a column of Toyopearl HW-65. The proteins were eluted with Tris–HCl 10 mM (pH 7.4) containing sucrose 0.25 M and 0.2% (w/w) Tween 80, and fractions of 1 ml were collected. Arrows at the top indicate deduced molecular weights obtained by Gel Filtration Molecular Weight Markers (Sigma and Amersham Pharmacia Biotech). Protein concentration (○) and enzyme activity (●) were determined as described in MATERIALS AND METHODS.

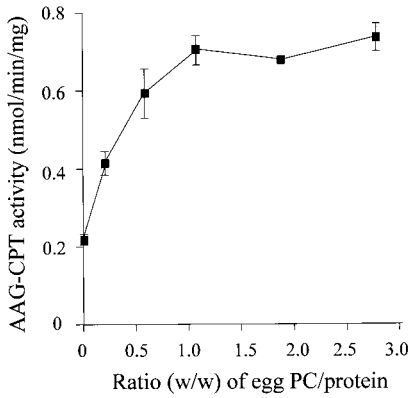

Fig. 2. Effects of Egg PC on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

The enzyme obtained after solubilization and sequential column chromatography on Toyopearl HW-65 and DEAE-Toyopearl 650 (30 μl) was combined with egg PC dissolved in Tris–HCl 50 mM (pH 7.4) and 0.2% (w/w) Tween 80 (150 μl). The ratios of egg PC to protein were as indicated. The enzyme reaction was carried out as described in MATERIALS AND METHODS. Data are expressed as mean ± S.D. of three determinants.

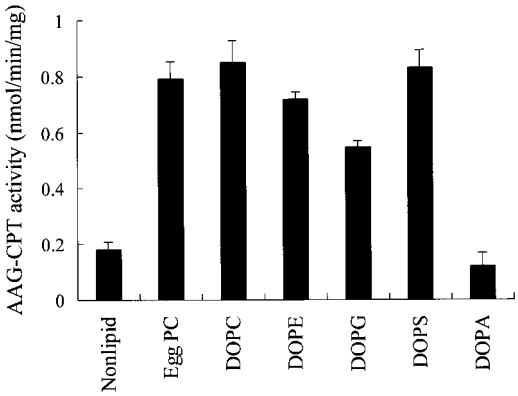

Fig. 3. Effects of a Series of Diacylphospholipids on Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

The enzyme obtained after solubilization and subsequent column chromatography on Toyopearl HW-65 and DEAE-Toyopearl 650 (30 μl) was combined with the individual diacylphospholipids dissolved in Tris–HCl 50 mM (pH 7.4) and 0.2% (w/w) Tween 80 (150 μl). The ratio of diacylphospholipid to the protein was maintained at 1.9. The enzyme reaction was carried out as described in MATERIALS AND METHODS. Data are expressed as mean ± S.D. of three determinants.

tained after solubilization and subsequent column chromatography at the same lipid/protein ratio (1.9, w/w), as shown in Fig. 3. DOPC, DOPE, DOPG, and DOPS also recovered the activity that decreased during the process of column chromatography. In contrast, DOPA showed almost no effect or a slightly inhibitory effect on the enzyme activity.

Effects of Concomitant Addition of Phospholipids on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

We further examined the effects of concomitantly added DOPA and DOPC on enzyme activity. When the DOPA/DOPC ratio was increased, enzyme activity once reactivated by DOPC was clearly inhibited at the ratio of more than 0.25 (w/w) and the reactivated enzyme activity was almost canceled at the (w/w) ratio of DOPA/DOPC of 1, as shown in

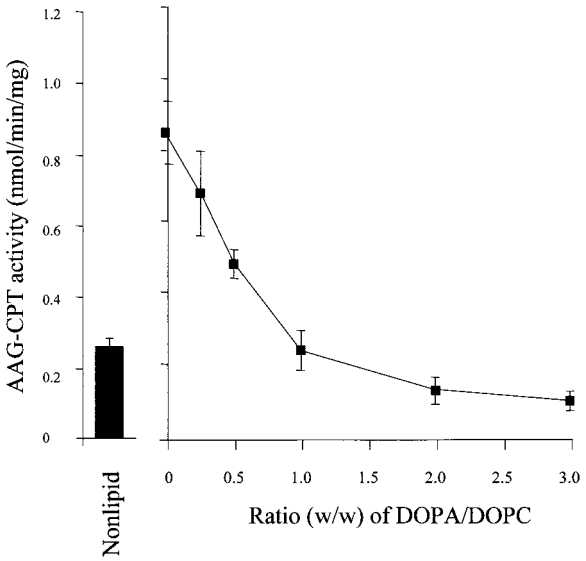

Fig. 4. Effects of Concomitant Addition of DOPA and DOPC on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

The enzyme obtained after solubilization and subsequent column chromatography on Toyopearl HW-65 and DEAE-Toyopearl 650 (30 μl) was combined with the mixture consisting of the indicated ratios of DOPA and DOPC dissolved in Tris–HCl 50 mM (pH 7.4) and 0.2% (w/w) Tween 80 (150 μl). The ratio of DOPC to protein was maintained at 1.9. The enzyme reaction was carried out as described in MATERIALS AND METHODS. Data are expressed as means ± S.D. of three determinants.

Fig. 4.

Effects of PA Endogeneously Produced by Phospholipase D on AAG-CPT Activity in Intact Microsomes

We examined whether endogenously generated PA showed the same inhibitory effect on the AAG-CPT decrease in intact microsomes. Since *S. chromofuscus* phospholipase D is used to increase the intracellular PA and examine the role of PA in cellular function,[7,8] we treated porcine microsomes with this phospholipase D to generate PA in the porcine microsomes. Microsomes treated with phospholipase D showed significantly decreased AAG-CPT activity, as compared with the control and heat-inactivated phospholipase D, as shown in Fig. 5. Accumulation of PA was detected in the microsomes treated with phospholipase D (data not shown). Since it was reported that unsaturated fatty acids such as oleic acid and arachidonic acid activate endogenous microsomal phospholipase D but that palmitic acid shows no effect,[9–12] we examined whether activation of endogenous phospholipase D by oleic acid affected the AAG-CPT activity of porcine spleen microsomes. Treatment of microsomes with oleic

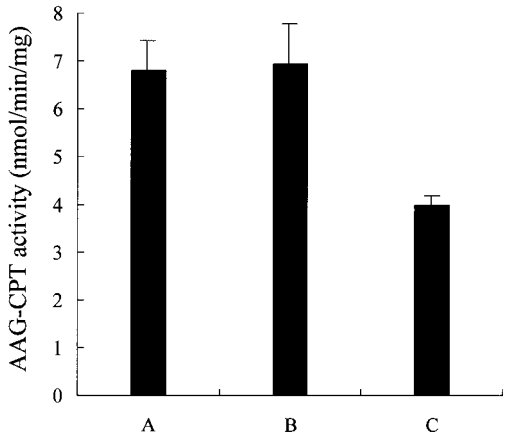

Fig. 5. Effects of Exogenously Added Phospholipase D on the Activity of AAG-CPT in Intact Microsomes

Porcine spleen microsomes (22 μg, 0.028 μmol phosphorus) were reconstituted in Tris–HCl 25 mM (pH 7.4) containing KCl 25 mM, $MgCl_2$ 2.5 mM, sucrose 0.2 M, EDTA 1 mM, DTT 2 mM and $CaCl_2$ 2 mM in a total volume of 100 μl. This mixture was incubated at 37°C for 30 min in the absence (A) or presence (C) of *S. chromofuscus* phospholipase D (10 μg, 0.75 units), and then AAG-CPT activity was determined according to the method described in MATERIALS AND METHODS. (B) Phospholipase D inactivated by heating at 100°C for 5 min was added to the mixture. Data are expressed as means ± S.D. of three determinants.

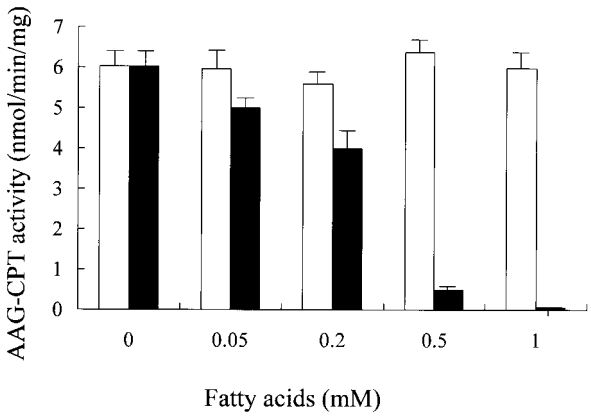

Fig. 6. Effects of Activation of Endogenous Phospholipase D on the Activity of AAG-CPT in Intact Microsomes

Porcine spleen microsomes (71 μg, 0.1 μmol phosphorus) were reconstituted in the solution consisting of β,β-dimethylglutarate Tris–HCl 25 mM (pH 6.5), $MgCl_2$ 2 mM, and propranolol 0.2 mM in the presence of sodium oleate 0.05–1 mM (■) or sodium palmitate (□) in a total volume of 100 μl. This mixture was incubated at 37°C for 60 min, and then AAG-CPT activity was determined according to the method described in MATERIALS AND METHODS. Data are expressed as means ± S.D. of three determinants.

acid resulted in a remarkable decrease in the AAG-CPT activity of porcine microsomes in a dose–dependent manner (Fig. 6). On the other hand, palmitic acid did not show any inhibitory effect, suggesting that the decrease in AAG-CPT activity resulted

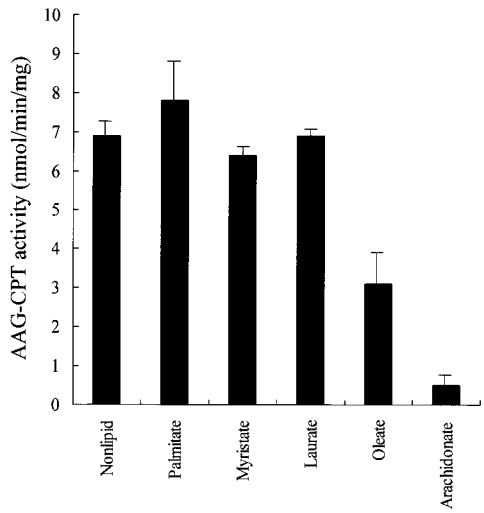

Fig. 7. Effects of Activation of Endogenous Phospholipase D by Various Fatty Acids on the Activity of AAG-CPT in Intact Microsomes

Porcine spleen microsomes (71 μg, 0.1 μmol phosphorus) was reconstituted in the solution consisting of β,β-dimethylglutarate Tris–HCl 25 mM (pH 6.5), $MgCl_2$ 2 mM, and propranolol 0.2 mM in the presence of 0.5 mM sodium palmitate, sodium myristrate, sodium laurate, sodium oleate, or sodium arachidonate in a total volume of 100 μl. This mixture was incubated at 37°C for 60 min, and then AAG-CPT activity was determined according to the method described in MATERIALS AND METHODS. Data are expressed as mean ± S.D. of three determinants.

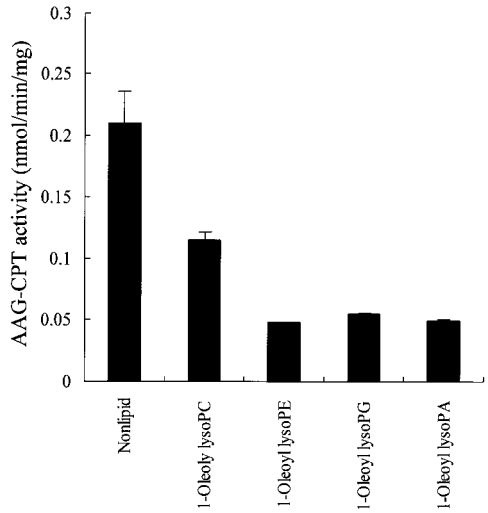

Fig. 8. Effects of a Series of Lysophospholipids on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

The enzyme obtained after solubilization and sequential column chromatoghraphy on Toyopearl HW-65 and DEAE-Toyopearl 650 (15 μl) was combined with the individual lysophospholipids dissolved in Tris–HCl 50 mM (pH 7.4) and 0.2% (w/w) Tween 80 (150 μl). The ratio of lysophospholipid to protein was maintained at 3.75. The enzyme reaction was carried out as described in MATERIALS AND METHODS. Data are expressed as means ± S.D. of three determinants.

from activation of endogenous phospholipase D of porcine spleen microsomes. The effects of various fatty acids on the activity of porcine spleen microsomes were also inverstigated (Fig. 7). Of the fatty acids tested, only oleic acid and arachidonic acid decreased the enzyme activity of porcine spleen microsomes. Arachidonic acid inhibited almost all the enzyme activity at concentrations as low as 0.5 mM. Arachidonic acid is reported to activate phospholipase D from microsomes of rat brain[10,11] and pig lung[12] at concentrations lower than those of oleic acid. Taken together, the activation of phospholipase D by these unsaturated fatty acids may be associated with the regulation of AAG-CPT activity.

Effects of Lysophospholipids on the Activity of Enzyme Obtained after Solubilization and Subsequent Column Chromatography

We examined the effects of lysophospholipids on the activity of the enzyme obtained after solubilization and subsequent column chromatography. As a result, all the tested lysophospholipids showed remarkable inhibitory effects on enzyme activity (Fig. 8). The inhibitory effect of MOPC on enzyme activity was much less than those of MOPE, MOPG, and MOPA at a fixed ratio of lysophospholipid to protein (3.75, w/w). These inhibitory effects of MOPC and MOPA were detected at the ratios (w/w) of lysophospholipid to protein down to 1, and MOPA showed a markedly higher inhibitory effect on enzyme activity than MOPC (data not shown).

We also examined whether phospholipase A_2, an enzyme that produces lysophospholipids from diacylphospholipids, affected AAG-CPT activity in intact microsomes from porcine spleen. The treatment of porcine spleen microsomes with phospholipase A_2 inhibited the enzyme activity in a dose–dependent manner (Fig. 9).

DISCUSSION

Although AAG-CPT activity has been reported in the microsomes of many mammalian tissues, difficulty in the solubilization of this enzyme made it impossible to characterize this enzyme in more detail. In this study, we succeeded in the solubilization of AAG-CPT from porcine spleen microsomes in an active form using digitonin. Further column chromatography of solubilized enzyme resulted in decreased enzyme activity, which was reactivated by

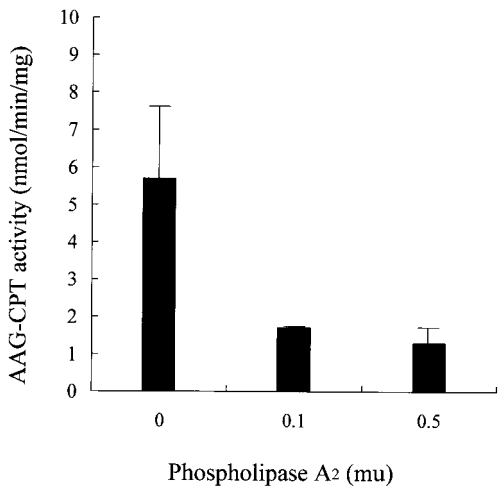

Fig. 9. Effects of Phospholipase A_2 on the Activity of Microsomal AAG-CPT

Porcine spleen microsomes (71.5 μg protein, 0.1 μmol phosphorus) were reconstituted in Tris–HCl 25 mM (pH 7.4) containing KCl 25 mM, $MgCl_2$ 2.5 mM, sucrose 0.2 M, and $CaCl_2$ 4 mM. Indicated amounts of *Naja naja* phospholipase A_2 were added to the mixture and incubated at 30°C for 60 min. One milliunit (mu) of phospholipase A_2 releases 1 nmol of fatty acid/min from L-α-phosphatidylcholine. After treatment of porcine microsomes with phospholipase A_2, AAG-CPT activity of the microsomes was determined according to the method described in MATERIALS AND METHODS. Data are expressed as mean ± S.D. of three determinants.

exogenously added phospholipids such as egg PC, DOPC, DOPE, DOPS, and DOPG. On the other hand, DOPA and lysophospholipids further inhibited enzyme activity. In addition, accumulation of PA and lysophospholipids in intact microsomes resulted in decreased enzyme activity. In this study, we showed the possibility that the environmental membrane phospholipids around the enzyme affected AAG-CPT activity.

Among the detergents tested, only a few were effective in the solubilization of this enzyme in an active form. Analysis of the enzyme solubilized with digitonin by gel-filtration chromatography indicated that it forms a complex sufficiently large to include other protein molecules and membrane phospholipids. A significant amount of phosphorus was detected in the active fraction obtained after gel-filtration column chromatography. Therefore it is suggested that the membrane phospholipids are necessary to maintain the enzyme in an active form. When this enzyme is solubilized with digitonin, the enzyme is removed from the membrane as a complex in which the structure necessary for enzyme reaction is retained. Therefore it is possible that the detergents causing inactivation of this enzyme bind to the sites on the enzyme molecule normally occupied by phospholipids.[13] Although the activity of enzyme solubilized with digitonin is relatively stable, remarkable loss of enzyme activity occurs after subsequent column chromatography. The decreased enzyme activity is partially recovered by exogenously added phospholipids necessary for maintaining enzyme activity during sequential column chromatography. The charge of the polar head groups of the phospholipids is not likely to be involved in the reactivation of the enzyme, because negatively charged phospholipids such as DOPG and DOPS also reactivate the enzyme, as well as noncharged phospholipids such as egg PC and DOPC. On the other hand, DOPA showed an inhibitory effect on the enzyme activity. In addition, DOPC-induced reactivation of the enzyme was competitively inhibited by DOPA, suggesting that PA caused inhibition of enzyme activity by interacting with specific binding sites necessary for the enzyme to remain in an active form. Similarly, inhibition of enzyme activity by lysophospholipids was also observed. MOPA showed greater inhibitory effects on enzyme activity than other lysophospholipids, for example, lysophosphatidylcholine, suggesting that polar head groups of lysophospholipids also play a role in enzyme inhibition. However, the possibility that inhibition by lysophospholipids occurs due to dilution of enzyme substrates cannot be ruled out.

PAF has been shown to cause phospholipase D activation, leading to production of PA in several different cell types such as neutrophils, macrophages, and monocytes.[14,15] In addition, neutrophils[16] and mesangial cells[17] respond to PAF with increased *de novo* phospholipid synthesis that contributes to PA mass accumulation. Therefore PA formation by PAF might create a negative feedback loop to maintain a constant PAF level. On the other hand, lysoPC is reported to stimulate phospholipase D activity in some cell types such as endothelial cells[18] and peritoneal macrophages.[19,20] It is thereby possible that lysoPC affects the production of PAF by increasing the PA level. Although further studies are required, the present results raise the possibility that AAG-CPT activity is regulated by the change in composition of PA and/or lysophospholipids among membrane phospholipids *in vivo*.

The effects of phospholipids on DG-CPT, similar enzyme to AAG-CPT, have been examined.[21–26] The results suggest that phospholipids play a role in protecting DG-CPT from inactivation by detergents. Although the effect of PA on mammalian DG-CPT has not been described, yeast DG-CPT, which shares

a homologous structure with that of mammalian DG-CPT,[27] is reported to be activated by PA with other phospholipids including lysophospholipids.[28] These different effects of phospholipids on both enzymes might provide additional evidence that AAG-CPT is a separate enzyme from DG-CPT, although we must await the isolation of the gene for AAG-CPT to understand the relationship of the two enzymes.

REFERENCES

1) Snyder, F. (1985) Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked choline-phospholipids. *Med. Res. Rev.*, **5**, 107–140.
2) Snyder, F. (1997) CDP-choline:alkylacetylglycerol cholinephospho-transferase catalyzes the final step in the de novo synthesis of platelet-activating factor. *Biochim. Biophys. Acta*, **1348**, 111–116.
3) Renooij, W. and Snyder, F. (1981) Biosynthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor and a hypotensive lipid) by cholinephosphotransferase in various rat tissues. *Biochim. Biophys. Acta*, **663**, 545–556.
4) Woodard, D. S., Lee, T.-C. and Snyder, F. (1987) The final step in the de novo biosynthesis of platelet-activating factor. Properties of a unique CDP-choline:1-alkyl-2-acetyl-sn-glycerol cholinephosphotransferase in microsomes from the renal inner medulla of rats. *J. Biol. Chem.*, **262**, 2520–2527.
5) Francescangeli, E. and Goracci, G. (1989) The de novo biosynthesis of platelet-activating factor in rat brain. *Biochem. Biophys. Res. Commun.*, **161**, 107–111.
6) Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipide extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911–917.
7) Metz, S. A. and Dunlop, M. (1990) Stimulation of insulin release by phospholipase D. A potential role for endogenous phosphatidic acid in pancreatic islet function. *Biochem. J.*, **270**, 427–435.
8) Siddiqui, R. A. and Exton, J. H. (1992) Oleate stimulation of diacylglycerol formation from phosphatidylcholine through effects on phospholipase D and phosphatidate phosphohydrolase. *Eur. J. Biochem.*, **210**, 601–607.
9) Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C. and Roth, M. G. (1996) Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. *J. Cell. Biol.*, **134**, 295–306.
10) Kobayashi, M. and Kanfer, J. N. (1987) Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.*, **48**, 1597–1603.
11) Chalifour, R. and Kanfer, J. N. (1982) Fatty acid activation and temperature perturbation of rat brain microsomal phospholipase D. *J Neurochem.*, **39**, 299–305.
12) Okamura, S. and Yamashita, S. (1994) Purification and characterization of phosphatidylcholine phospholipase D from pig lung. *J. Biol. Chem.*, **49**, 31207–31213.
13) Hjelmeland, L. M. and Chrambach, A. (1984) Solubilization of functional membrane proteins. *Methods Enzymol.*, **104**, 305–318.
14) Kanaho, Y., Kanoh, H., Saitoh, K. and Nozawa, Y. (1991) Phospholipase D activation by platelet-activating factor, leukotriene B4, and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. Phospholipase D activation is involved in enzyme release. *J. Immunol.*, **146**, 3536–3541.
15) Balsinde, J. and Mollinedo, F. (1991) Platelet-activating factor synergizes with phorbol myristate acetate in activating phospholipase D in the human promonocytic cell line U937. Evidence for different mechanisms of activation. *J. Biol. Chem.*, **266**, 18726–18730.
16) Uhing, R. J., Prpic, V., Hollenbach, P. W. and Adams, D. O. (1989) Involvement of protein kinase C in platelet-activating factor-stimulated diacylglycerol accumulation in murine peritoneal macrophages. *J. Biol. Chem.*, **264**, 9224–9230.
17) Tou, J., Jeter, R. J., Dola, C. P. and Venkatesh, S. (1991) Accumulation of phosphatidic acid mass and increased de novo synthesis of glycerolipids in platelet-activating-factor-activated human neutrophils. *Biochem. J.*, **280**, 625–629.
18) Kester, M. (1993) Platelet-activating factor stimulates phosphatidic acid formation in cultured rat mesangial cells: roles of phospholipase D, diglyceride kinase, and de novo phospholipid synthesis. *J. Cell. Physiol.*, **156**, 317–325.
19) Cox, D. A. and Cohen, M. L. (1996) Lysophosphatidylcholine stimulates phospholipase D in human coronary endothelial cells: role of PKC. *Am. J. Physiol.*, **271**, 1706–1710.
20) Izumi, T. and Shimizu, T. (1995) Platelet-activating factor receptor: gene expression and signal transduction. *Biochim. Biophys. Acta*, **1259**, 317–333.
21) Gómez-Muñoz, A., O'Brien, L., Hundal, R. and Steinbrecher, U. P. (1999) Lysophosphatidylcholine stimulates phospholipase D activity in mouse peritoneal macrophages. *J. Lipid Res.*, **40**, 988–993.
22) Cornell, R. and MacLennan, D. H. (1985) Solubilization and reconstitution of cholinephospho-

transferase from sarcoplasmic reticulum: stabilization of solubilized enzyme by diacylglycerol and glycerol. *Biochim. Biophys. Acta*, **821**, 97–105.

23) O, K. M. and Choy, P. C. (1990) Solubilization and partial purification of cholinephosphotransferase in hamster tissues. *Lipids*, **25**, 122–124.

24) Ishidate, K., Matsuo, R. and Nakazawa, Y. (1993) CDPcholine: 1,2-diacylglycerol cholinephosphotransferase from rat liver microsomes. I. Solubilization and characterization of the partially purified enzyme and the possible existence of an endogenous inhibitor. *Lipids*, **28**, 89–96.

25) Bru, R., Blochliger, E. and Luisi, P. L. (1993) *sn*-1,2-Diacylglycerol cholinephosphotransferase from pig liver: mixed micellar assay and kinetic analysis of the partially pure enzyme. *Arch. Biochem. Biophys.*, **307**, 295–303.

26) Henneberry, A. L., Wistow, G. and McMaster, C. R. (2000) Cloning, genomic organization, and characterization of a human cholinephosphotransferase. *J. Biol. Chem.*, **275**, 29808–29815.

27) Henneberry, A. L. and McMaster, C. R. (1999) Cloning and expression of a human choline/ethanolaminephosphotransferase: synthesis of phosphatidylcholine and phosphatidylethanolamine. *Biochem. J.*, **339**, 291–298.

28) McMaster, C. R., Morash, S. C. and Bell, R. M. (1996) Phospholipid and cation activation of chimaeric choline/ethanolamine phosphotransferases. *Biochem. J.*, **313**, 729–735.