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# Conversion of Glycosylphosphatidylinositol (GPI)-Anchored Alkaline Phosphatase by GPI-PLD

**Young Girl Moon[1], Hyun Jung Lee[1], Mee Ree Kim[2] , Pyung-Keun Myung[1], Soo-Young Park[1] and Dai-Eun Sok[1*]**

*[1]College of Pharmacy and [2]Department of Food and Nutrition, Chungnam National University, Taejon 305-764, Korea*

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Enzymatic conversion of brain glycosylphosphatidylinositol-linked alkaline phosphatase (GPI-AP), amphiphilic, was examined. When GPI-AP was incubated with glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD), a negligible conversion of GPI-AP to hydrophilic form was observed. The inclusion of monoacylglycerols enhanced the enzymatic conversion, although the action of monoacylglycerols differed greatly according to the size of acyl group; the enzymatic conversion was enhanced considerably in the presence of monoacylglycerols possessing acyl group of longer chain length ($C_{10}$-$C_{18}$), while monoacylglycerols with acyl moiety of shorter length ($C_4$-$C_8$) did fail to augment the enzymatic conversion. Noteworthy, monooleoylglycerol was much more effective than the other monoacylglycerols in promoting the enzymatic conversion, indicating a beneficial role of the unsaturation in acyl chain. Meanwhile, ionic amphiphiles such as monohexadecyllysophosphatidylcholine and palmitoylcarnitine decreased the enzymatic conversion of GPI-AP in a concentration-dependent manner, with monohexadecyllysophosphatidylcholine being more inhibitory than palmitoylcarnitine. Separately, when GPI-AP was exposed to various oxidants prior to the incubation with GPI-PLD, a remarkable decrease of the enzymatic conversion was observed with hypochlorite and peroxynitrite generators, but not $H_2O_2$. In further study, hypochlorite was found to inactivate GPI-PLD at low concentrations (3~100 μM). From these results, it is suggested that the enzymatic conversion of GPI-AP by GPI-PLD may be regulated *in vivo* system.

**Key words :** GPI-PLD, Phosphatase, Enhancement, Inhibition, Inactivation

## INTRODUCTION

There are various types of phosphatases (Holander, 1971), responsible for the hydrolysis of p-nitrophenylphosphate, which are widely distributed in tissues of vertebrates. Membrane-bound types of phosphatase and several types of phosphatase appearing in the cytosolic subcellular fraction have been described in brain tissue (Brunel *et al.*, 1973; Cathala *et al.*, 1975; Li and Chan, 1981). Most of membrane-bound alkaline phosphatase activity is embedded in membranes as a glycosylphosphatidylinositol (GPI)-anchored protein. Also, in cytosolic fraction, there are GPI-anchored alkaline phosphatase, amphiphilic, along with corresponding hydrophilic forms (Billington *et al.*, 1980; Deng *et al.*, 1996; Lee *et al.*, 1998).

Alkaline phosphatse is known to hydrolyze many types of phosphomonoesters (Brunel *et al.*, 1973; Irino *et al.*, 1994). Especially, the phosphatase from kidney membranes or intestinal membranes was observed to express a higher Vm value for phosphocholine than p-nitrophenylphosphate. In brain homogenate, a phosphatase activity responsible for the hydrolysis of p-nitrophenylphosphate and phosphocholine at alkaline pH was detected (Kanfer and McCartney, 1986). Thus, it was suggested that one of functions of alkaline phosphatase in brain tissue might be the conversion of phosphocholine to choline, a precursor for the synthesis of acetylcholine. In this respect, the fate of GPI-anchored alkaline phosphatase has been of a particular interest.

Previously, there have been reports (Low, 1989; Low and Huang, 1991) that GPI-anchored proteins are substrates for GPI-PLD, which is a hydrolase capable of removing phosphatidic acid from GPI-anchors. Concomitantly, the enzyme participates in the alteration of molecular form of GPI-anchored proteins (Low, 1989; Nosjean *et al.*, 1997). Further, once released from GPI-anchors, amphiphilic, the hydrophilic form could be free to move intracellularly, but more susceptible to the intracellular degradation, enzymatic or non-enzymatic. Since the GPI-PLD is only active against glycosylphos-

*Correspondence to*: Dai-Eun Sok, College of Pharmacy, Chungnam National University, Gung-Dong, Yuseong-Gu, Taejon 305-764, Korea
E-mail : daesok@hanbat.chungnam.ac.kr

phatidylinositol anchors solubilized in the detergent, the determination of GPI-PLD activity has been carried out in the presence of non-ionic detergents such as Triton X-100 or Nonidet P-40 (Low and Prasad, 1988; Low and Huang, 1993). Meanwhile, ionic detergents expressed a negative effect on the activity of GPI-PLD (Low and Huang, 1993; Lee *et al.*, 1998). From these observations, it is conceivable that brain GPI-PLD-catalyzed conversion of GPI-anchored alkaline phosphatase (Amp-Ap) to Hyd-AP may be affected by endogenous amphiphiles such as monoacylglycerols or lysophospholipids. However, there is no extensive study on the regulatory role of endogenous amphiphiles in the enzymatic conversion of Amp-AP. Earlier studies (Low and Prasad, 1988) had indicated that GPI-PLD was one of $Zn^{2+}$-metallohydrolases. Since $Zn^{2+}$-metalloenzymes generally contain histidine residue, oxidants-labile, coordinated with $Zn^{2+}$ atom, it is likely that GPI-PLD may be one of enzymes susceptible to oxidants. Despite this possibility, there is no report on the possible negative regulation of GPI-PLD activity by cellular oxidants, generated in brain tissue subjected to oxidative stress (Packer, 1988; Kim and Kwon, 1999). In this study, we attempted to examine the possible regulatory factors to affect GPI-PLD-catalyzed conversion of Amp-AP to Hyd-AP.

## MATERIALS AND METHODS

### Materials

1-Hexadecyl-2-hydroxy-sn-glycero-3-phosphocholine (monohexadecyllysophosphatidylcholine), palmitoylcarnitine, p-nitrophenylphosphate, concanavalin A sepharose, DEAE cellulose, Triton X-100, monoacylglycerols, 3-morpholinosydnonimine and α-methyl mannoside were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Triton X-114 was from Fluka Chemical Co. (Buchs, Switzerland). Other reagents used were of analytical grade.

#### Preparation of GPI-PLD from bovine brain

GPI-PLD was partially purified according to the procedures reported previously (Hoener *et al.*, 1990; Lee *et al.*, 1998). A fresh bovine brain (200 g) was extensively washed with 20 mM Tris buffer, pH 7.4, and homogenized in 1,800 ml of cold 20 mM Tris buffer (pH 7.0). The supernatant after centrifugation (25,000 g, 30 min) of brain homogenate was loaded onto DEAE cellulose column (3.7×20 cm), equilibrated with 20 mM Tris buffer (pH 7.0). The column was washed first with 200 ml of the above equilibrium buffer, and then with 200 ml of the same buffer containing 50 mM NaCl. Finally, the enzyme activity was eluted with the equilibrium buffer containing 400 mM NaCl.

#### Preparation of amphiphilic form of alkaline phosphatase (Amp-AP)

Amp-AP was isolated from the cytosol fraction of brain homogenateas as previously described for the isolation of amphiphilic form of $Zn^{2+}$-glycerophosphocholine cholinephosphodiesterase (Lee *et al.*, 1998); the supernatant from the centrifugation (25,000 g, 30 min) of brain homogenate was applied onto DEAE cellulose column (3.7×20 cm), which was washed with 20 mM Tris buffer (pH 7.0), and then the same buffer containing 50 mM NaCl. Finally, Amp-AP activity was desorbed from the column with the same buffer containing 400 mM NaCl, and further purified using concanavalin A-sepharose column (0.7×3 cm), which was eluted with 0.2 M α-methyl mannoside.

#### Assay of alkaline phosphatase

The activity of the phosphatase was determined by measuring the amount of p-nitrophenol produced from the hydrolysis of p-nitrophenylphosphate (p-NP) in 1 ml of 0.1 M Tris buffer (pH 9) containing 2 mM p-nitrophenylphosphate (Brunel et al., 1973; Kim *et al.*, 1998). One unit of enzyme activity was expressed in μmole of p-nitrophenol produced per min.

#### Effect of monoacylglycerols and monoalkylglycerols on the GPI-PLD-catalyzed conversion of Amp-AP

GPL-PLD-mediated conversion of Amp-AP to Hyd-AP was determined by incubating GPI-PLD (1.1 μg protein) with Amp-AP (1 milli unit) in 200 μl of 20 mM Tris buffer (pH 7.0) containing Triton X-100 (0.03 %) or each amphiphile as a detergent at 38°C. After 1 hr, the reaction mixture was mixed with 2% Triton X-114 (800 μl), and then the mixture was subjected to Triton X-114 phase separation. An aliquot (300 μl) in the aqueous phase was taken for the assay of Hyd-Ap activity. The amount of Hyd-AP generated from Amp-AP in the presence of monoacylglycerols or monoalkylglycerols was expressed as a percentage among total activity of Hyd-AP produced in the presence of 0.03 % Triton X-100. Data, presented as % of control value, are the mean±S.D. of three sets.

#### Inhibitory effect of lysophosphatidylcholine or palmitoylcarnitine on GPI-PLD-cayalyzed conversion

The inhibitory effect of monohexadecyllysophosphatidylcholine or palmitoylcarnitine was examined by including each inhibitor of various concentrations (10-300 μM) in the reaction mixture for the assay of GPI-PLD-catalyzed conversion of Amp-AP. The $IC_{50}$ value was indicated as the concentration of the inhibitor to reduce the enzymatic conversion in the absence of inhibitor (control) by 50%. Data, presented as % of

control value, are the mean ± S.D. of three sets.

### Effect of oxidants on the activity of GPI-PLD

The GPI-PLD (1.1 μg protein) was exposed to $H_2O_2$ (0.1 mM), hypochlorite (0.1 mM) or 3-morpholino-sydnonimine (0.1 mM) in 200 μl of 20 mM Tris buffer, pH 7.0 at 38°C for 30 min. Then, Amp-AP (1 milli unit) and 4 μl of monooleoylglycerol (15 mM) were added to the reaction mixture. After 1 hr incubation, the aliquot (300 μl) was taken for the assay of Hyd-AP released. Separately, the GPI-PLD was exposed to sodium hypochlorite (0-100 μM), and the remaining activity was determined as described above.

## RESULTS

Previously, it had been reported that in contrast to an inhibitory action of ionic detergents (Low and Huang, 1993), monomyristoylglycerol, non-ionic, expressed an activatory effect on GPI-PLD-catalyzed conversion of GPI-anchored $Zn^{2+}$-glycerophosphocholine cholinephosphodiesterase in the assay employing Triton X-100 as a detergent (Lee *et al.*, 1998). Based on these observations, we examined the direct effect of various endogenous amphiphiles on the enzymatic conversion of GPI-anchored alkaline phosphatase (Amp-AP). Since a preliminary evaluation indicated that a remarkable conversion of Amp-AP into Hyd-AP was observed with monoacylglycerols (data not shown), monoacylglycerols possessing an acyl moiety of a different size ($C_4$-$C_{18}$) were examined for the ability to enhance the GPI-PLD-catalyzed conversion of Amp-AP. In this experiment, Amp-AP was incubated with brain GPI-PLD for 1 hr at 38° C in the buffer containing each monoacylglycerol at 0.3 mM. The enzymatic conversion of Amp-AP to Hyd-AP in the presence of the respective amphiphile was determined and compared with that achieved in the presence of 0.03% Triton X-100. While the enzymatic conversion of Amp-AP was negligible in the incubation containing monobutyrylglycerol or monooctanoylglycrol at 0.3 mM, there was a remarkable conversion in the presence of monoacylglycerols bearing decanoyl to palmitoyl moiety (Fig. 1). There was a size-dependent effect of acyl group on the enzymatic conversion of Amp-AP. The enhancing effect of monoacylglycerols was augmented with increasing length of acyl group up to 12 carbon atoms, but the extention of chain length over 14 carbon atoms led to a reduction in the enhancing effect of monoacylglycerols; there seemed to be an optimal size of acyl group ($C_{12}$-$C_{14}$) for the effective conversion of Amp-AP by GPI-PLD. In the subsequent study, a concentration-dependent effect of monoacylglycerols on the GPI-PLD-catalyzed conversion was examined. As demonstrated in Fig. 2, monolaurylglycerol enhanced the enzymatic conversion of Amp-AP in a concentration-dependent manner up to 0.3 mM, exhibiting a half maximal effect ($EC_{50}$) at 0.05 mM. The enhancement by monolaurylglycerol at 0.3 mM was about 50% of that accomplished with 0.03% Triton X-100. However, the increase of monolaurylglycerol concentration over 0.3 mM led to a dramatic decrease of conversion rate, and the conversion at 2.0 mM decreased to below 10% level. In the related study, where the effect of monododecylglycerol, a monoalkylglycerol, was investigated, it was found that the enzymatic conversion of Amp-AP was augmented by monododecylglycerol in a concentration-dependent

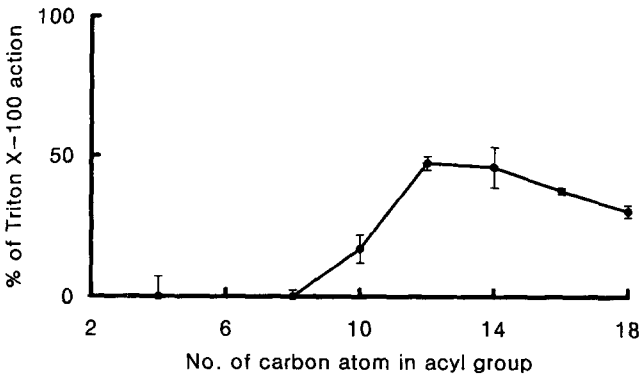


**Fig. 1.** Effect of monoacylglycerols with acyl group of a different size on GPI-PLD-mediated conversion of Amp-AP. Amp-AP (1 milli unit) was incubated with brain GPI-PLD (1.1 μg protein) in 200 μl of 20 mM Tris buffer, pH 7.0 containing the respective monoacylglycerol at 0.3 mM. The formation of Hyd-AP was determined as described in Methods, and the effect of monoacylglycerol on the enzymatic conversion was expressed as a relative percentage among the effect achieved with 0.03 % Triton X-100. Data are the mean±S.D. of three sets.

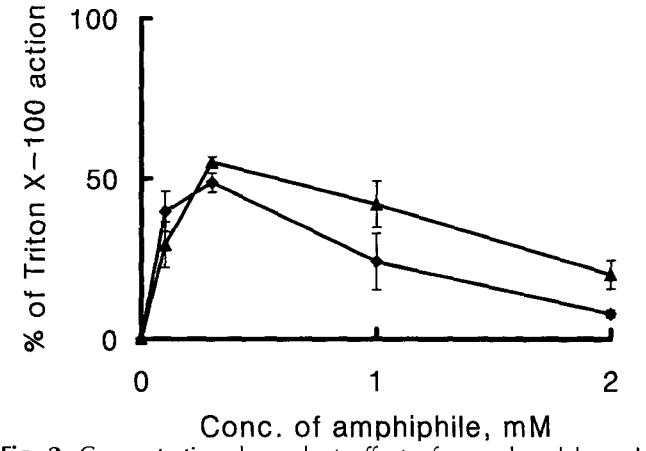


**Fig. 2.** Concentration-dependent effect of monolaurylglycerol or monododecylglycerol on GPI-PLD-mediated conversion of Amp-AP. Amp-AP (1 milli unit) was incubated with brain GPI-PLD (1.1 μg protein) in 200 μl of 20 mM Tris buffer, pH 7.0 containing monolaurylglycerol (◆-◆) or monododecylglycerol (▲-▲) of various concentrations (0~2 mM). Values are mean± S.D. (n=3).

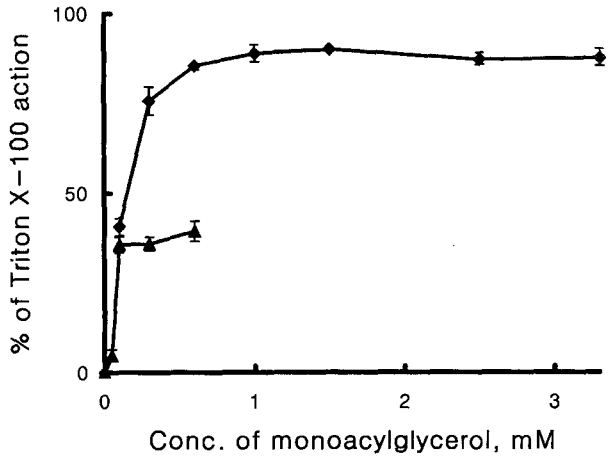


**Fig. 3.** Effect of monopalmitoylglycerol or monooleoylglycerol on GPI-PLD-mediated conversion of Amp-AP. ▲-▲, monopalmitoylglycerol; ◆-◆, monooleoylglycerol

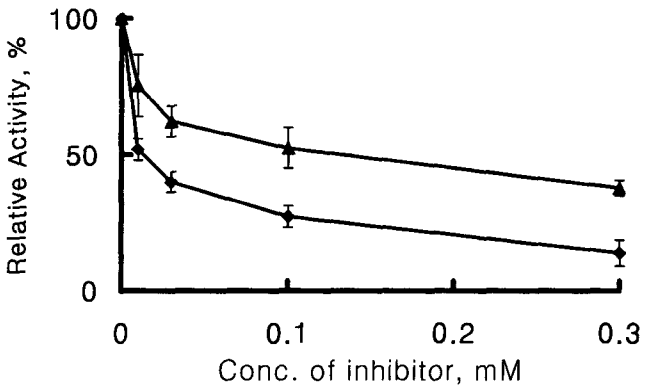


**Fig. 4.** Inhibitory effect of ionic amphiphiles on GPI-PLD-mediated conversion of Amp-AP. The enzymatic conversion of Amp-AP was performed in the presence or absence of each inhibitor in 200 μl of 20 mM Tris buffer, pH 7.0 containing 0.3 mM monooleoylglycerol as a detergent. Values are mean±S.D. (n=3).

manner with an $EC_{50}$ value of approximately 0.1 mM, but over 0.3 mM, its enhancing effect was diminished, similar to the above observation with monolaurylglycerol; although monolaurylglycerol appeared to be slightly more effective than monododecylglycerol at lower concentrations (0~0.3 mM), the enhancing effect at higher concentrations (>0.3 mM) was greater with monododecylglycerol. Next, the effect of monoacylglycerols containing a long acyl group ($C_{16}$-$C_{18}$) on enzymatic conversion was examined. As shown in Fig. 3, the enhancing effect of monopalmitoylglycerol was augmented up to 0.1 mM, but above the concentration a further enhancement was not expressed probably due to a limited solubility. This result led us to examine the importance of the hydrophilicity in the acyl group. For this purpose, monooleoylglycerol, possessing 11-cis-unsaturated acyl moiety ($C_{18}$), was examined for the ability to enhance the enzymatic conversion of Amp-AP. As exhibited in Fig. 3, monooleoylglycerol enhanced the GPI-PLD-catalyzed conversion of Amp-AP up to 1.0 mM. Moreover, its enhancing effect showed no decline over a broad range of high concentrations (1.5 ~3.3 mM). Noteworthy, the maximal enhancing effect of monooleoylglycerol was close to 90% of that achievable with 0.03 % Triton X-100.

Since ionic detergents had been observed to exert an inhibitory effect on GPI-PLD activity (Lee *et al.*, 1998), it was supposed that the GPI-PLD-catalyzed conversion of Amp-AP might be reduced in the presence of endogenous ionic amphiphiles such as lysophosphatidylcholine or palmitoylcarnitine. In this respect, the inhibitory effect of these ionic amphiphiles on the enzymatic conversion of Amp-AP was examined in the assay system employing monooleoylglycerol as a detergent. When Amp-AP was exposed to GPI-PLD in the presence of monohexadecyllysophosphatidylcholine of various concentrations (0.01~0.3 mM) in the buffer containing 0.3 mM monooleoylglycerol as a detergent, it was found (Fig. 4) that monohexadecyllysophosphatidylcholine inhibited the enzymatic conversion in a concentration-dependent manner with an $IC_{50}$ value of around 15 μM. In additional experiment, palmitoylcarnitine was also found to show an inhibition of the enzymatic conversion of Amp-AP with an $IC_{50}$ value of around 150 μM. Thus, there was a difference of inhibitory action between two zwitter-ionic amphiphiles.

In the following experiment, the possible inactivation of GPI-PLD, requiring $Zn^{2+}$ for the activity, by endogenous oxidants was explored, since $Zn^{2+}$-requiring hydrolases had been known to be susceptible to the mixed function oxidation. For this purpose, the GPI-PLD was preincubated with each oxidant system, and the remaining activity, responsible for the conversion of Amp-AP into Hyd-AP, was determined. Table 1 indicates that the activity of GPI-PLD decreased markedly during the incubation with hypochlorite or 3-morpholinosydnonimine, HOONO genearator, which had a negligible effect on the activity of phosphatase activity at concentrations used. Meanwhile, GPI-PLD activity was not affected remarkably by $H_2O_2$. In further study (Fig. 5), HOCl was found to reduce the activity of GPI-PLD in a

**Table 1.** Effect of oxidants on GPI-PLD-mediated conversion of Amp-AP

| Oxidant | Concentration, μM | Relative Activity (%) |
|---|---|---|
| control | | 100 |
| $H_2O_2$ | 100 | 88.9±13.1 |
| NaOCl | 100 | 32.9±10.4* |
| 3-Morpholinosydnon-imine | 100 | 51.6±6.4 |

Values are the mean±S.D. *, 10 min preincubation.

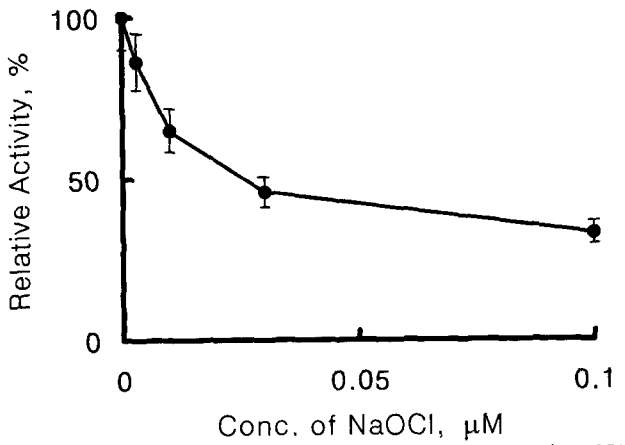


**Fig. 5.** Effect of hypochlorite on GPI-PLD activity. The GPI-PLD (1.1 μg protein) was exposed to hypochlorite of various concentrations (3-100 μM) in 200 μl of 20 mM Tris buffer (pH 7.0) for 10 min, and then Amp-AP (1 milli unit) and 4 μl of monooleoylglycerol (15 mM) were added to the reaction mixture. The remaining activity of GPI-PLD was expressed as a percentage of control. Values are mean±S.D. (n=3).

concentration-dependent manner (3~100 μM). The $IC_{50}$ value was estimated to be as low as 25 μM under condition used.

## DISCUSSION

Present results demonstrate that the GPI-PLD-catalyzed conversion of Amp-AP to Hyd-AP was substantially enhanced in the presence of some monoacylglycerols. These non-ionic amphiphiles could replace synthetic non-ionic detergents, which have been used for the solubilization of GPI-anchors as substrates, in the assay of GPI-PLD activity (Low, 1989; Koelisch *et al.*, 1994; Rhode *et al.*, 1995). The size of the acyl chain seems to govern a role of monoacylglycerols in the conversion of Amp-AP. The enhancing effect increased proportionally according to the length of acyl chain ($C_8$-$C_{12}$), with the maximal effect being observed with monolaurylglycerol or monomyristoylglycerol. However, the elongation of the carbon atom chain over 14 carbon atoms rather resulted in a corresponding decrease of enhancing effect, which might be ascribed partially to the limitation of solubility. From these results, it is supposed that the enhancing effect of monoacylglycerols may be related to their ability of micelle formation (Gennis, 1989), which is determined by the hydrophilicity and hydrophobicity of acyl chain. The higher efficacy of monooleoylglycerol, compared with monopalmitoylglycerol, might be explained by higher hydrophilicity. On the other hand, the decline in enhancing effect of monoacylglycerols at high concentrations is not explained properly by present results. In contrast to monopalmitoylglycerol, monolaurylglycerol seemed to show a narrow range of concentrations for a maximal enhancing effect, implying that monolaurylglycerol at high concentrations might exert an inhibitory effect on the activity of GPI-PLD. It is of note that despite a structural difference, monododecylglycerol and monolaurylglycerl are similar to each other in the capability to enhance the enzymatic conversion of GPI-AP, implying that the size and hydrophobicity of acyl group may be important for the optimal funtion. However, It is likely that monododecylglycerol may be more effective than monolaurylglycerol *in vivo* system, since the former is more resistant to intracellular degradation than the latter. In support of this, a recent report (Warne *et al.*, 1995) demonstrates that a substantial amount of monododecylglycerol accumulates in some cells. In this respect, monoalkylglycerol, possessing an unsaturated alkyl moiety, could be more effective in enhancing GPI-PLD activity. Taken together, it is proposed that monoacylglycerols or monoalkylglycerols at physiological concentrations may play a positive role in GPI-PLD-catalyzed conversion of Amp-AP into Hyd-AP *in vivo* system.

Meanwhile, the conversion by GPI-PLD was strongly inhibited in the presence of monohexadecyllysophosphatidylcholine. A similar degree of inhibition was also expressed by monopalmitoyllysophosphatidylcholine (data not shown). The $IC_{50}$ value of lysophosphatidylcholine is close to a physiological concentration (-50 μM) of lysophosphatidylcholine (Wong *et al.*, 1998). Since lysophosphatidylcholine is one of metabolites during cellular activation (Billah and Anthes, 1990), it is possible to suppose that lysophosphatidylcholine may be one of endogenous ionic amphiphiles, which may play a negative role in the GPI-PLD-mediated conversion of Amp-AP. The selective action of lysophosphatidylcholine may be supported by the observation that despite the same ionic property, palmitoylcarnitine, which is not directly involved in cell activation, had a much smaller inhibitory effect on the GPI-PLD action. Based on these observations, the GPI-PLD-catalyzed conversion of Amp-AP to Hyd-AP in vivo system might be regulated by at least two types of endogenous amphiphiles, inhibitory or activatory. An additional factor to affect the enzymatic conversion of Amp-AP may be the myeloperoxidase/$H_2O_2$ system as suggested from HOCl-mediated deactivation of brain GPI-PLD. Since it has been evidenced (Hazen et al., 1996) that HOCl is produced during cellular activation such as phagocytosis, it is possible that GPI-PLD-catalyzed conversion of Amp-AP may be diminished during cellular activation. In this respect, it is conceivable that the generaton of HOCl during cellular activation, taken together with the increase of $PLA_2$ action, could lead to the reduction in the enzymatic conversion of Amp-AP. Additionally, peroxynitrite is also suggested to contribute to the loss

of GPI-PLD activity, which needs further study.

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