Biological Functions of a Novel Lipid Mediator, Cyclic Phosphatidic Acid (cPA)

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Abstract: Cyclic phosphatidic acid (cPA) possessing phosphate at *sn*-2 and -3 positions of glycerol is a unique analog of lysophosphatidic acid (LPA). The biosynthesis and functions of cPA as a novel lipid mediator have been studied.

The biosynthetic enzyme activity of cPA was detected in sera from rat, bovine and human using lysophosphatidylcholine as a substrate. The reaction is considered to be catalyzed by a phospholipase D -like enzyme. The naturally occurring cPA and some of their analogs showed a variety of biological activities.

cPA showed an inhibitory effect on cdc25 phosphatase *in vitro*, while no effect on activities of cdks. It was also shown that dephosphorylated (active) form of cdk2 decreased when cPA was added into cultures of human fibroblast cells caused a decrease in dephosphorylated (active) form of cdk2. These results suggested that suppression of cell proliferation by cPA is caused by the inhibition of cdc25 phosphatase.

LPA induced tumor cell invasion, whereas cPA suppressed the LPA-induced invasion. cPA containing C16:0 was most potent in inhibiting tumor cell invasion (transcellular migration) in an *in vitro* system as compared with other molecular.

These findings support the idea that cPA is formed as a physiological constituent, and functions as one of the bioactive lipids in mammalian cells.

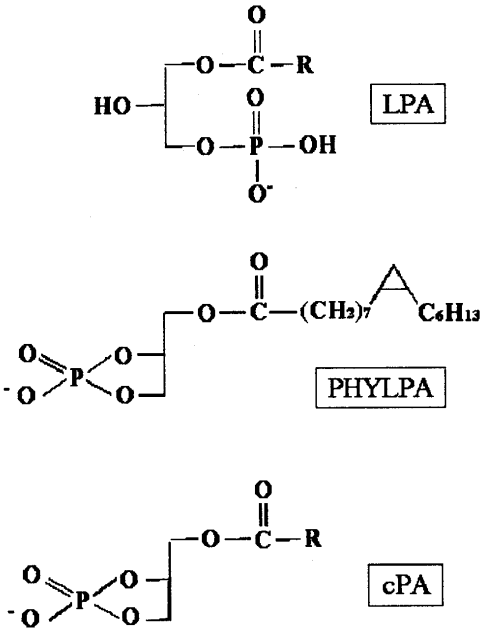

Fig. 1 Structures of LPA, PHYLPA and cPA. "R" represents a hydrocarbon chain.

1. Introduction

Cyclic phosphatidic acid, which has a cyclic phosphate at *sn*-2 and -3 positions of glycerol carbons, is a unique analog of lysophosphatidic acid (LPA) (Fig. 1). It was found and isolated originally from myxoamoebae of a true slime mold, *Physarum polycephalum*, and designated as PHYLPA or cPA [1]. cPA was also detected as a naturally occurring substance of human serum [2] and aqueous humor of rabbit eyes [3].

cPA was demonstrated to have various biological activities including i) an antiproliferative effect on eukaryotic cell cycle [4, 5], ii) regulation of Ca^{2+} release [4,5], iii) regulation of actin rearrangement [5], and iv) inhibition of tumor cell invasion [6]. To clarify the physiological significance of cPA we have investigated its biosynthesis and signaling pathway for the cPA-induced inhibition of cell proliferation. The inhibitory effects of tumor cell invasion by cPA have been also investigated.

2. Enzymatic formation of cPA

In course of studying the degradation products from lysophosphatidylcholine (LPC) by cabbage leaf phospholipase D (PLD), Long *et al.* found the formation of cPA-like product as well as LPA more than 30 years ago [7]. This can be explained by intramolecular transphosphorylation with the hydroxyl group at *sn*-2 position of LPC as shown in Fig. 2. The efficiency of transphosphatidylation as compared to hydrolysis is known to be different among PLD enzymes.

The formation of cPA from LPC by PLD derived from *Actinomadura* sp. No. 362, which is known to possess a high activity of transphosphatidylation rather than hydrolysis, was examined as compared with that by PLD from *Streptomyces chromofuscus* with

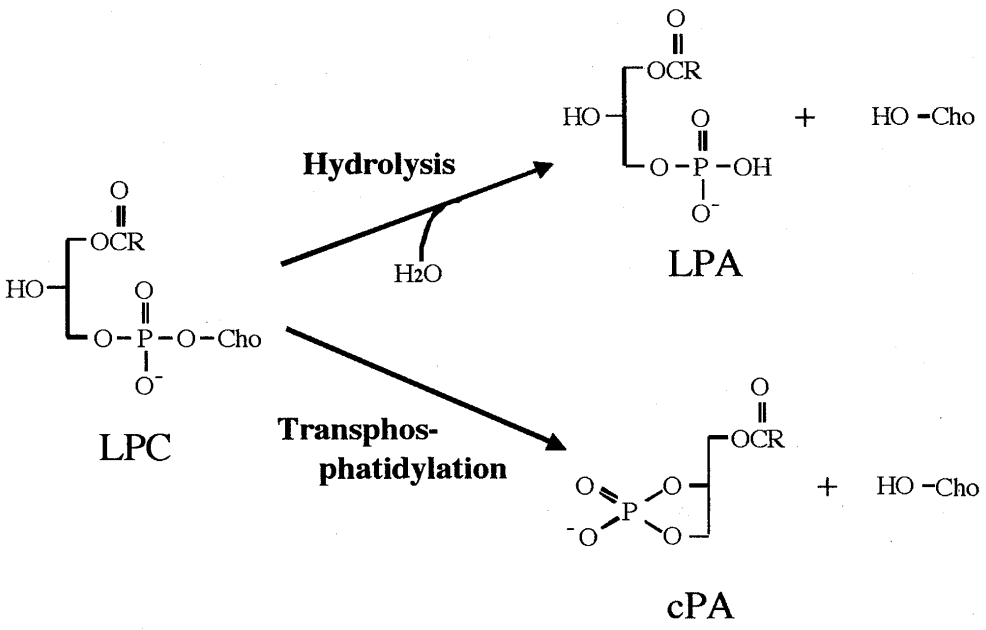

Fig. 2 Reactions of transphosphatidylation and hydrolysis catalyzed by phospholipase D (PLD). PLD catalyzes the hydrolysis of phospholipids (e.g. PC) to produce phosphatidic acid (PA) and their respective polar head groups (e.g. choline). The enzyme attacks the substrate to form a transient phosphatidyl-PLD intermediate which is able to react either with water as an acceptor to produce PA or with a primary alcohol to generate phosphatidylalchol. This reaction, known as transphosphatidylation, is a unique feature of PLD. When lysophosphatidylcholine (LPC) is used as the substrate, intramolecular transphosphorylation possibly occurred with the hydroxyl group at *sn*-2 position of LPC.

a low transphosphatidylation activity. The *Actinomadura* PLD enzyme produced almost exclusively a product which co-migrated with an authentic standard of cPA on a thin-layer chromatography plate. In contrast, the *Streptomyces* PLD enzyme formed LPA, but not cPA. The product generated by Actinomadura PLD was identified to be cPA by mass spectrometry analysis and bioassay of stress fiber formation in fibroblasts. We developed a method of enzymatic synthesis of cPA using *Actinomadura* PLD to prepare structural derivatives of cPA as an alternative to chemical synthesis.

Next, we tried to detect a biosynthetic enzyme activity of cPA in mammalian cell homogenates and tissues with reference to the assay conditions of *Actinomadura* PLD. So far, cPA has been produced by incubating LPC with rat brain homogenate and with serum from rat, bovine and human. On the other hand, our preliminary data showed that a significant amount of cPA was detected in rat and porcine brain. Recently, we have also demonstrated a neurotrophic effect of cPA on primary cultures of hippocampal neurons of rat. These findings support the idea that cPA may play an important role in the neuronal functions of mammalian brains.

3. Suppression of cell proliferation by cPA

Different types of LPA receptors have been detected and their genes cloned. Some of them were also activated by cPA [5]. It was demonstrated previously that cPA caused an increase in cAMP [4, 5], which was completely abolished when the rise in intracellular Ca^{2+} was blocked, suggesting that a Ca^{2+}-activated adenylate cyclase coupled with cell surface receptor was involved [5]. Then, the elevation of cAMP could inhibit mitogen-activated protein (MAP) kinase activity by PKA-mediated phosphorylation to cause the antimitogenic action. In addition to the cell surface receptors, here, we propose the possibility that cPA may interact directly with a cell cycle regulatory protein to inhibit cell proliferation.

Flow cytometric analysis showed that cPA induced a decrease in the number of S-phase cells, and increase in that of G_1-phase cells, suggesting that cPA may affect a G_1/S check point of cell cycle. Cdk2 is known to be activated by dephosphorylation, which is catalyzed by cdc25 phosphatase, to go though the G_1/S check point. cPA showed a marked inhibitory effect on the cdc25 phosphatase activity *in vitro*. Furthermore, it was shown that supplementation of cPA into cultures of human fibroblast cells caused a decrease in the dephosphorylated (active) form of cdk2. These results suggested that the suppression of cell proliferation by cPA is attributable to the inhibition of cdc25 phosphatase as shown in Fig. 3. Since cdc25 phosphatase is located in nucleus and cytosol, there should be a transport pathway which is involved in the internalization of exogenously added cPA into

cells.

4. Inhibition of tumor cell invasion by cPA

We used *in vitro* invasion assay system. First, rat mesotherial cell monolayer was prepared, and then cancer cells, such as MM1 cells, which are the cells of a highly invasive clone of rat ascites hepatoma cells, were seeded over the monolayer and cultured in medium containing compounds to be tested. 24 hours later, the monolayer was fixed with formalin, and the number of penetrated tumor cell colonies was counted by phase contrast microscopy.

When tumor cells were incubated with fetal calf serum (FCS), cell invasion was induced significantly. This effect of FCS can be completely substituted by 25 μM C18:1-LPA, indicating that the invasion inducible factor in FCS is attributed primarily to LPA.

Next, we examined the effect of cPA on LPA-induced tumor invasion. When a variety of cPA analogs were added simultaneously into the medium with LPA, an inhibitory effect was observed. The final concentration of cPA in the medium was 25μM. Among these cPA analogs, a molecular species of C16:0-containing cPA showed the most potent inhibition as compared with other molecular species containing C12:0, C16:1, C18:1 or C20:4.

To examine if the effects of cPA were specific for invasion by MM1 cells or not, we tested other cell lines, mouse melanoma cells, human pancreatic adenocarcinoma cells, and human lung cancer cells. Invasion by these cells was also inhibited by cPA to an extent similar to that of MM1 cells. Furthermore, invasion by human fibrosarcoma cells which do not require either FCS or LPA, was also suppressed by cPA, although to a lesser extent.

We adopted the system of experimental model of metastasis of B16 mouse melanoma cell lines to examine the effects of cPA on metastasis *in vivo*. A single injection of cPA concomitantly with melanoma cells into the tail vein of C57BL mice suppressed pulmonary metastasis when assayed 3 weeks after the injection. Approximately 50% and 90% suppression was observed with 4 and 8 μg/mouse of cPA, respectively.

The molecular mechanism by which cPA inhibits LPA-induced invasion is not fully understood, but our data support the following idea as shown in Fig. 3: LPA binding to its receptor causes Rho activation *via* $G_{12/13}$ activation. This Rho activation leads to rapid remodeling of the actin cytoskeleton. On the other hand, cPA caused an increase in intracellular cAMP, which was abolished when the elevation of intracellular Ca^{2+} was blocked, suggesting that a Ca^{2+}-activated adenylate cyclase coupled with cell surface

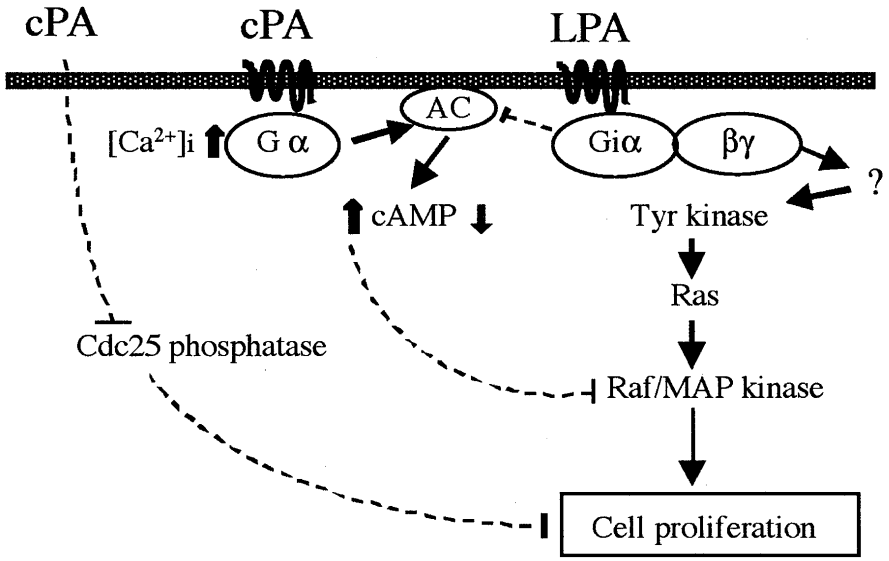

Fig. 3 Possible signaling pathways for LPA/cPA action on fibroblasts. Mitogenic action of LPA is shown to be mediated by activation of the cell surface receptor coupled with G proteins and the ras-raf-ERK pathway, which involves the βγ subunit of Gi. LPA decreases cAMP levels, while cPA causes an increase in cAMP, which is inhibited when the rise in $[Ca^{2+}]_i$ is blocked [5]. The elevation of cAMP could inhibit the activation of raf/MAP kinase. On the other hand, cPA inhibits directly cdc25 phosphatase, and cells were arrested at GI Phase. Dashed lines represent inhibitory effects.

receptor should be involved. Then, the elevation of cAMP could inhibit Rho/MAPkinase activity by the PKA-mediated phosphorylation.

5. Conclusion

The enzyme activity involved in cPA synthesis from lysophosphatidylcholine was detected in rat brain homogenate and sera of rat, bovine and human. It is considered that the reaction is transphosphatidylation catalyzed by a phospholipase D-like enzyme. cPA inhibited directly cdc25 phosphatase. Its antiproliferative effect on human fibroblast cells appears to be ascribed to the suppression of cdc25 phosphatase. cPA showed the inhibitory effect of tumor cell invasion. These findings support the idea that cPA is produced as a physiological constituent, and acts as one of the bioactive lipids in mammalian cells.

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