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STIMULATION OF Gq PROTEIN-COUPLED SIGNAL TRANSDUCTION BY CYCLIC PHOSPHATIDIC ACID (cPA) AND ACTIVATION OF PROTEIN KINASE C *IN VITRO*

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Key words: cyclic phophatidic acid (cPA) / PHYLPA / protein kinase C (PKC)/Gq protein/ signal transduction

Abbreviations: LPA; lysophosphatidic acid, cPA; cyclic lysophosphatidic acid, DG; diacyl-glycerol, PKC; protein kinase C, cPKC; conventional PKC, nPKC; novel PKC, PS; phophatidylserine, TPA; tetradecanoylphorbol 13-acetate, bFGF; basic fibroblast growth factor

Abstract

A unique cyclic phosphatidic acid (cPA), named PHYLPA, which was originally isolated from myxoamoebae of a true slime mold, *Physarum polycephalum*, and composed of cyclic phosphate and cyclopropane-containing hexadecanoic acid (1-3), was shown to stimulate the Gq protein-coupled signal transduction system and activity of protein kinase C (PKC) *in vitro*. When fibroblast cells were treated with PHYLPA, phosphoinositide hydrolysis and Ca^{2+} mobilization were immediately occurred, and a specific PKC substrate, MARKS, was markedly phosphorylated. Some derivatives of PHYLPA synthesized chemically also showed the obvious stimulatory effects on MARKS phosphorylation. These results show that cPA may act on a cell surface, specific receptor which activates Gq protein, a member of trimeric G protein, and phosphoinositide breakdown and PKC activation occur subsequently.

While, cPA obviously stimulated purified PKC activity, and the extent of activation was much higher than that by usual lysophosphatidic acid (LPA) or diacylglycerol (DG). This indicates the possibility that the cPA may be generated by the lipolytic action from plasma membrane and activates PKC directly in the cell.

cPA activated both conventional PKC (cPKC) and novel PKC (nPKC), former of which has Ca^{2+}-regulatory domain, and latter one has not, at different extents. One of the stereoisomers of natural PHYLPA, assigned as sodium 3-*O*-[(9'S,10'R)-9',10'-methanohexadecanoyl]-*sn*-glycerol 1,2-cyclic phosphate, showed an apparently high stimulatory activity on these enzymes.

Introduction

A unique *Physarum* lysophosphatidic acid, PHYLPA, has been isolated as a specific inhibitor of DNA polymerase (1). PHYLPA, composed of cyclic phosphate and cyclopropane-containing hexadecanoic acid, and its derivatives which are generally refferred to as a cyclic phosphatidic acid (cPA) selectively inhibit a family of DNA polymerase α, *e.g.* DNA polymerase α, δ and ε (3). Inhibition of the enzyme is largely the result of

competition between the lipid and the template DNA (1).

We tested the effects of PHYLPA on cultured cells. It inhibits proliferation of human fibroblasts in a reversible manner, and an increase in intracellular cAMP is considered to be critical for the antiproliferative action by PHYLPA (4).

A usual LPA which has a common fatty acid at C-1 and a phosphate at C-3 position of glycerol backbone shows further low or no effects on DNA polymerases (1,3). LPA elicits a variety of cellular responses in various types of cells (5-8), and it acts as a hormone-like lipid mediator that induces mitogenic resposes in the cells through a pertussis toxin-sensitive signaling pathway (9-14). LPA is proved to be produced in activated platelets from phosphatidylinositol (PI) and phosphatidic acid (PA) during blood clotting (15-17) and in growth factor-stimulated fibroblasts (18). And it is considered to play an essential role in wound healing and tissue regeneration in the minute environment (7).

These differences in the effects by cPA and LPA on cell proliferation were demonstrated to depend on the structure composed of cyclic phosphate or non-cyclic phosphate (manuscript in preparation). They may bind to different receptors and activate different types of G-protein. But, both cPA and LPA elicit phosphatidylinositol turnover in the same manner, suggesting the phospholipase C (PLC)-PKC pathway is not necessary for evoking a proliferative, or antiproliferative response caused by these lipids in the cell. PLC-PKC pathway is considered not to be involved in the proliferative process that is regulated by LPA and cPA.

Although the biological significance of activation of PKC by both LPA and cPA is not clear but the activation mechanism of PKC by these substances is to be clarified. In this report, we demonstrated that cPA activate PLC-PKC-pathway *in vivo*, and it also apparently elicits the activity of purified protein kinase, cPKC and nPKC, in *vitro*.

Materials and Methods

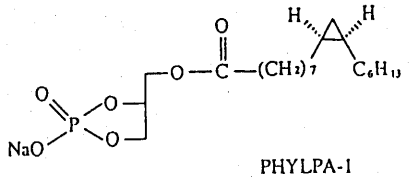
Chemicals. Fatty acid-free bovine serum albumin (FA-free BSA), DNase-free BSA, 1-palmitoyl or 1-oleoyl lysophosphatidic acid (LPA), diacylglycerol (DG), 2-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma, phosphatidylserine (PS) was from Avanti, and [γ-^{32}P]ATP was a product of Dupont-New England Nuclear. Oligopeptide substrate for PKC assay was $MBP_{1\text{-}14}$ (QKRPSQRSKYL), corresponding to the amino acid sequence 4-14 of myelin basic protein (19) was synthesized using a peptide synthesizer (model 430A, Applied Biosystems).

Chemical synthesis of PHYLPA and its derivatives. PHYLPA and its derivatives shown in Fig.1 were synthesized according to the procedure reported before (20).

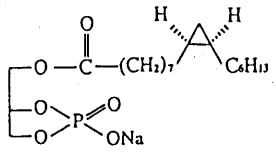
Determination of myo-inositol 1,4,5-tris-phosphate (IP_3). Cells in logarithmic phase were labeled with [^{3}H] myo-inositol (4mCi/ml) for 48 hrs. After the cells reached the late-log phase, they were treated with 10mM PHYLPA with 10mM LiCl. The reaction was stopped by addition of ice-cold 10% TCA and centrifuged. The supernatants were washed with diethylether and were neutralized with Tris. Then, IP_3 was separated by HPLC and determined according to Tilly *et al.*(21).

Expression and purification of cPKC and nPKC. Expression and purification of the enzymes expressed in COS1 cells were carried out as previously described (22-26).

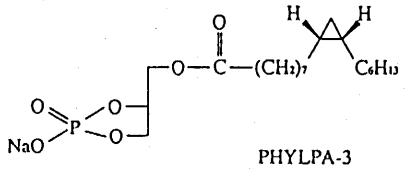
Protein kinase assay. The standard PKC assay was performed as described (21). The enzyme was incubated at 0℃ for 60 min in 40 μl of the assay mixture containing 20mM Tris-HCl (pH 7.5), 5mM magnesium diacetate, 50mM $CaCl_2$, 50μg/ml $MBP_{4\text{-}14}$, 10mg/ml leupeptin, with or without 10μg/ml PS, 25ng/ml TPA or 4-20μg/ml lipid. Added 10μl of ATP solution containing 20mM ATP and 0.5mCi of [γ-^{32}P]ATP, the mixture was incubated at 30℃ for 10 min. Aliquots (40μl) were spotted onto 20-mm squares



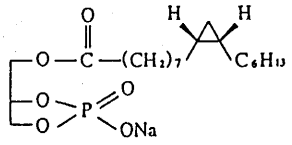
PHYLPA-1



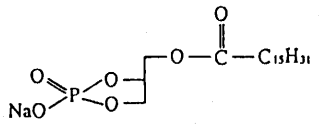
PHYLPA-2



PHYLPA-3



PHYLPA-4



PHYLPA-5
(Pal-cPA)

Fig.1. Chemically synthesized derivatives of cPA, PHYLPA-1 and Pal-cPA are naturally occurring cPA(1,34)

of phosphocellulose paper (Whatman P81), washed, dried, and radioactivities were measured.

Phosphorylation of 80 kD protein. Procedure was basically according to van Corven *et al.* (11). Human fibroblasts,TIG-3 cells were routinely cultured in a serum-free medium, MCDB-104, supplemented with 25ng/ml bFGF, insulin (5μg/ml), transferrin (5μg/ml) and dexamethasone (10ng/ml) (27). They were transferred into phosphate-free MCDB-104 medium as above and containing 0.5mCi/ml of ^{32}Pi, then incubated at 37℃ for 60 min. Lipid or TPA was added and incubated further for 15 min. Cells were washed and lysed in 100ml of SDS sample buffer and heated at 100℃ for 5 min. Then two-dimensional electrophoresis was performed by the procedure of O'Farrell (28). In the first dimension, isoelectric focusing in 4% ampholytes pH 3.5-5.0 plus 1% ampholytes pH 3.5-10.0 was used and in the second dimension, SDS-polyacrylamide gel electrophoresis (10% gel) was performed. And the spot of 80 kD protein was cut and the radioactivity was measured in toluene-based liquid scintilation fluid.

Results

Stimulation of Gq protein-coupled, phosphoinositide (PI) /Ca^{2+}/PKC pathway. We examined the possible correlation of PI/Ca^{2+}/PKC signaling pathway in PHYLPA-induced antiproliferative effect on the fibroblast

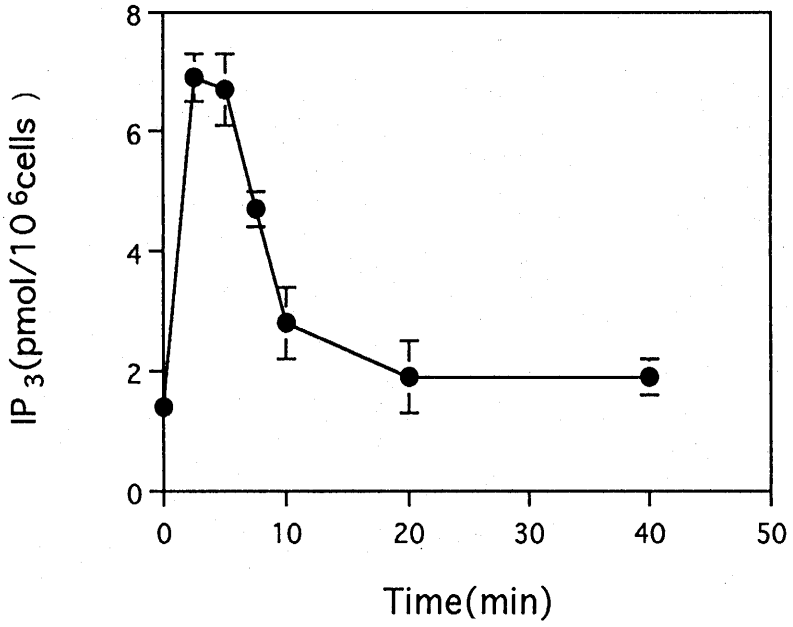


Fig.2. IP_3 formation caused by PHYLPA-1 in TIG-3cells. [H^3]Inositol-prelabeled cells were incubated with 10μM PHYLPA-1 for various periods and IP_3 was determined.

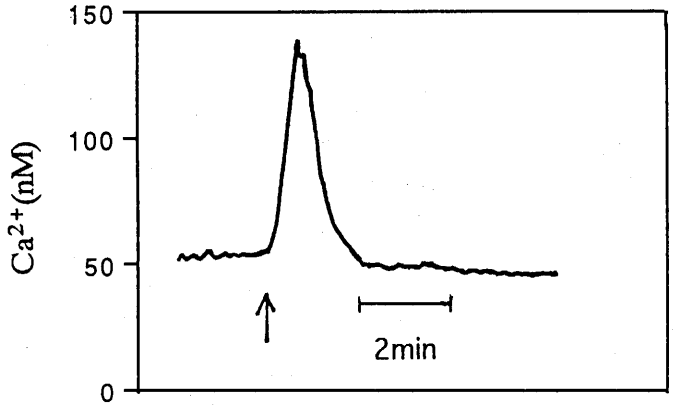


Fig.3. Ca^{2+} mobilization caused by PHYLPA-1 in TIG-3 cells. Cells were stimulated with 10μM PHYLPA-1 as indicated(arrow), and fluorescence was monitored.

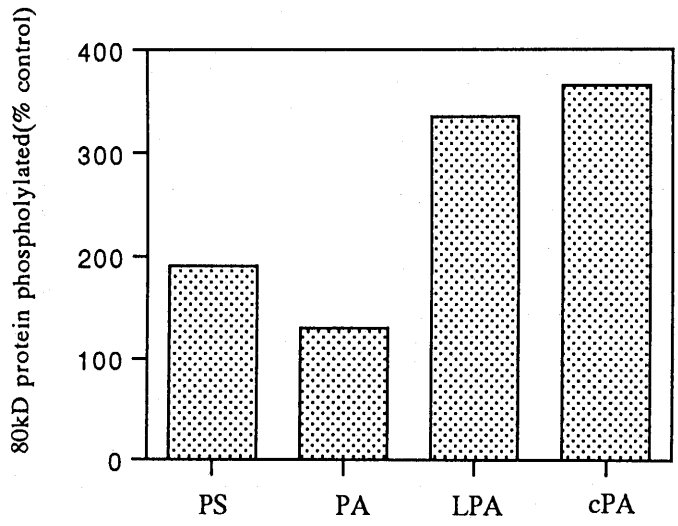


Fig.4. Stimulation of phosphorylation of 80kD protein. TIG-3 cells were labeled with ^{32}Pi in the presence or absence of test substances, and 80kD protein was separated and analyzed as described in the text.

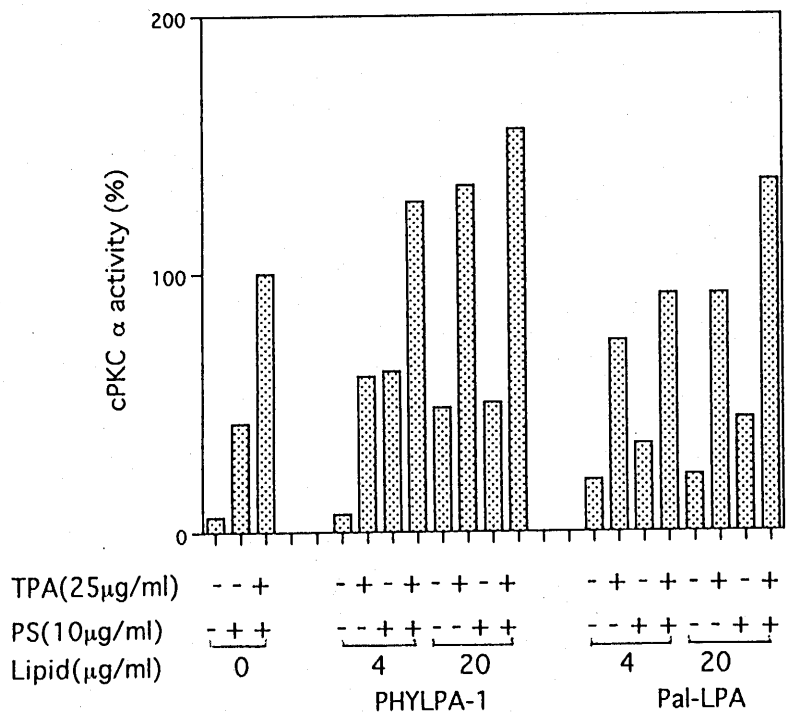


Fig.5. Effects of PHYLPA or Pal-LPA on cPKC α activity. cPKC α activity was determined with test substances as described in Materials and Methods.

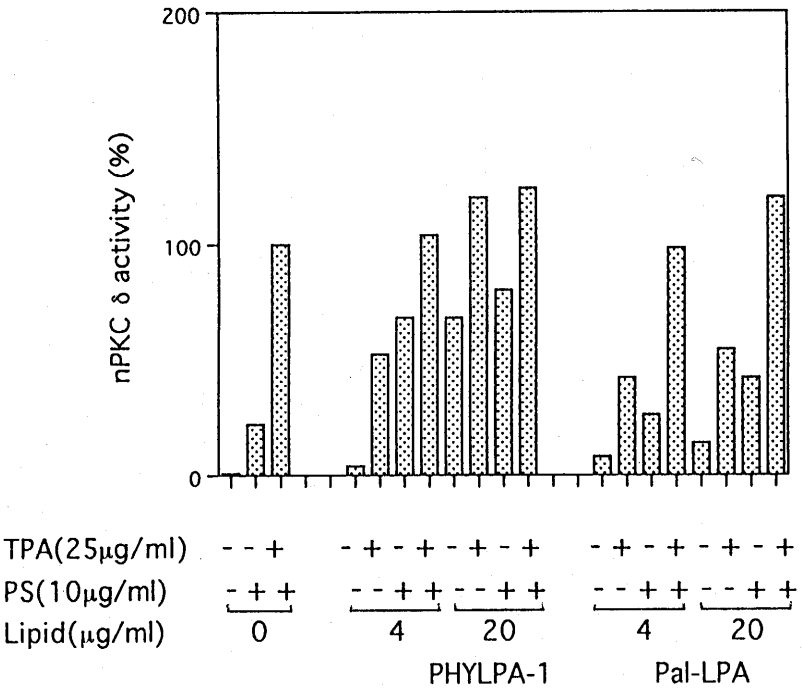


Fig.6. Effects of PHYLPA-1 or Pal-LPA on nPKC δ activity. nPKC δ activity was determined with test substances as described in the text.

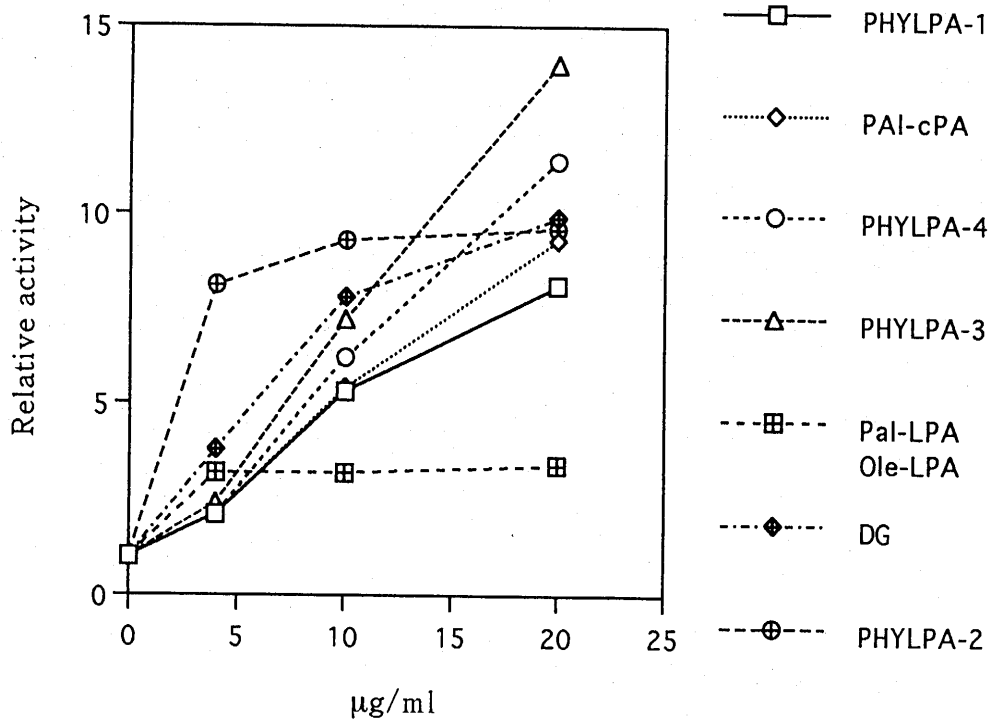


Fig.7. Effects of some derivatives of cPA and LPA on cPKC α activity.

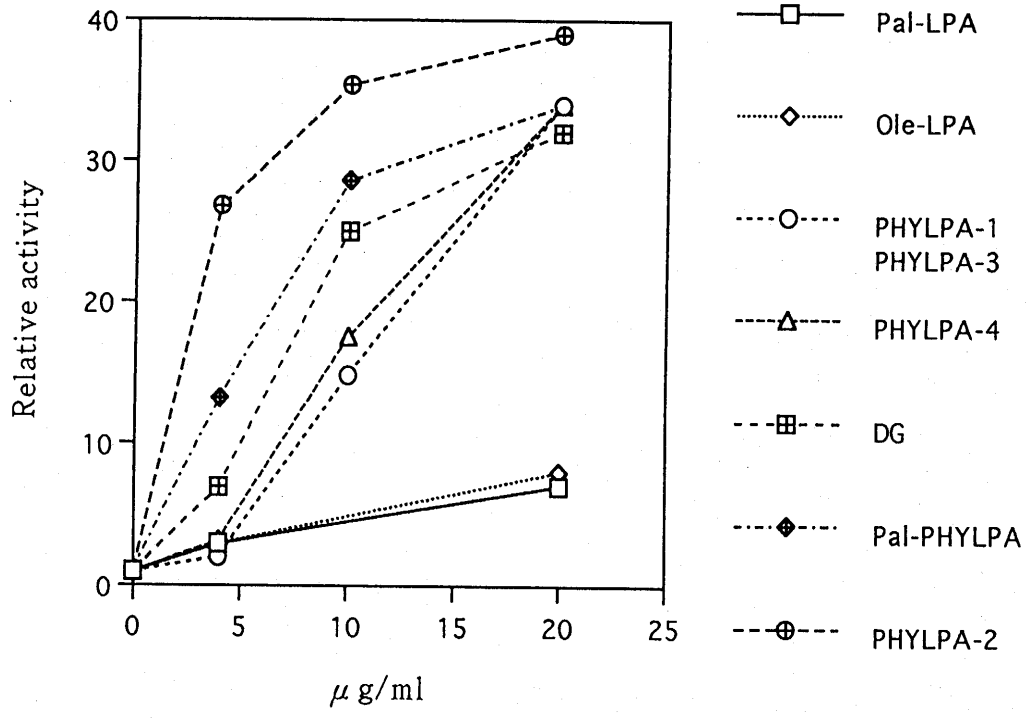


FIg.8. Effects of some derivatives of cPA and LPA on nPKC δ activity.

and we demonstrated that PHYLPA-1 elicits an increase in cellular IP_3 just after the addition of PHYLPA-1, and the response to PHYLPA is dose-dependent with a half maximal effect at about 5μg/ml (approx.10μM) (Fig.2). And PHYLPA-1 immediately rised in intracellular free Ca^{2+} concentration (Fig.3). So, PHYLPA-1 may activate phospholipase C through Gq protein, then PKC may be activated.

In order to determine whether PHYLPA activates PKC *in vivo*, the phosphorylation of an 80kD cellular protein which is a specific substrate of PKC (29) was examined. PHYLPA-1 stimulated the phosphorylation of the 80kD cellular protein in a few minutes as shown in Fig.4, then PHYLPA is proved to induce *in vivo* PKC activation. Ordinal LPAs also stimulate the phosphorylation of this protein in the same extent like the phorbol ester TPA (11).

Stimulation of PKC activity in vitro. PHYLPA and known LPAs obviously stimulated purified PKC like an *in vivo* stimulation. We tested the effects of PHYLPA on conventional PKC (cPKC) which has Ca^{2+}-regulatory domain (Fig.5) and on novel PKC (nPKC) which has no Ca^{2+}-regulatory domain (Fig.6). When PHYLPA or LPA alone was added to the reaction mixture, a dose-dependent stimulation was observed. When PS or/and TPA were added moreover, additive stimulation was observed as shown in Figs.5 and 6.

Some derivatives shown in Fig. 1 were chemically synthesized and their effects on PKCs were compared to reveal the relationship of stimulatory activity and molecular structure (Figs.7 and 8). At a low concentration (4μg/ml), PHYLPA-2, assigned as sodium 3-*O*-[(9'S,10'R)-9',10'- methanohexa-decanoyl]-*sn*-glycerol 1,2-cyclic phosphate, showed an apparently high stimulatory activity. At 4μg/ml of PHYLPA-2 alone,8-times activation for cPKC and 27-times activation for nPKC were observed. At the same concentration, DG which is known as a potent activator of PKC showed 4-times activation for cPKC, and 7-times for nPKC. So, PHYLPA-2 is strong stimulator for PKCs, especially for nPKC. At 20μg/ml, PHYLPA derivatives showed almost the same extents of stimulation: 8-14 times stimulation for cPKC, and 34-39 times stimulation for nPKC. But at 20 μg/ml, relatively low stimulation was observed (3.5-times for cPKC, and 7-times for nPKC).

These results show that the cyclic phosphate is effective for the strong stimulation of PKCs, and stereochemistry of fatty acid moiety is also important but not necessary for the enzyme stimulation.

To know the mechanism of signal transduction underlying the antiproliferative activation of cPA, in this study we examined the effect of cPA on PI/Ca^{2+}/PKC pathway. cPA caused an activation of this pathway which is mediated by Gq protein (Fig.9). PI/Ca^{2+}/PKC pathway is also stimulated by known LPA (7, 9, 11, 12) which is known as a potent mitogen of the fibroblast cells. These findings suggest that the PI/Ca^{2+}/PKC pathway is not necessary for evoking a proliferative, or antiproliferative response caused by these lipids in the cell.

Discussion

In this report, we showed the activation of PI/Ca^{2+}/PKC pathway by cPA derivatives and also show the direct activation of PKC by these lipids. PKC is thought to play pivotal roles in cellular signal transduction, coupled with receptor-mediated lipid metabolism (30-32), and is dependent on Ca^{2+}, phospholipid and diacylglycerol. PKC is also activated by tumor-promoting phorbol esters which mimic various cellular functions (30,31).

The PKC family is now classified into two categories, the conventional (cPKC) and the novel (nPKC), on the basis of their domain structures (24-26). The major difference between these groups is the presence or absence of the putative Ca^{2+}-regulatory domain designated as C2. cPKC group (α, β Ⅰ, βⅡ, γ) contains the C2 domain and is activated in the presence of Ca^{2+}, phospholipids and DG or phorbol ester. nPKC group (δ,

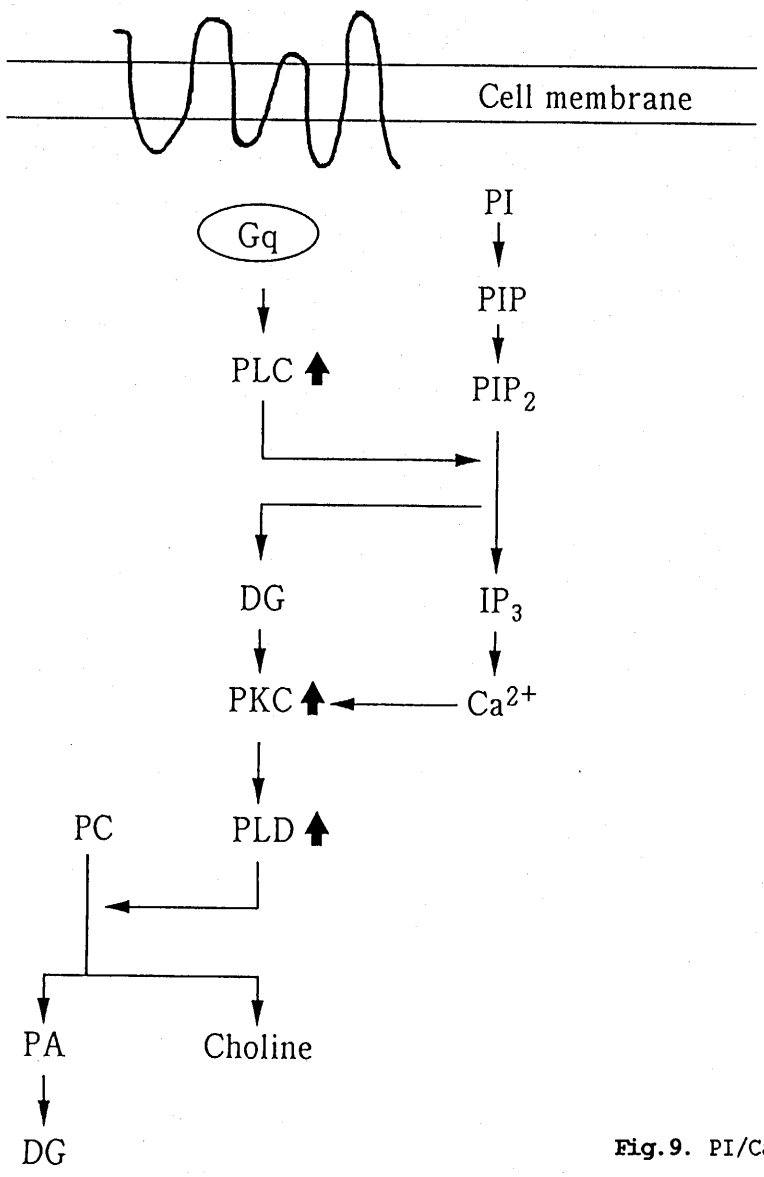


Fig. 9. PI/Ca^{2+}/PKC pathway stimulated by cPA.

ε, ζ, η) doesn't contain the C2 domain and thus is expected to have Ca^{2+}-independent activity. These enzymes have been considered to act as a key enzyme at an essential step of regulation of cell proliferation. But from the results that the activation and inhibition of cell proliferation caused by LPA and cPA, respectively, rather adenylate cyclase than PKC is supposed to play an critical role in the regulation of cell proliferation.

cPA stimulates PI/Ca^{2+}/PKC pathway in the cell, and also stimulates the purified PKC activity. cPA or LPA may reacts with the specific receptor exist on the cell surface and activate coupled G-protein (33). On the other hand, cPA or LPA may be produced by hydrolysis of membrane lipids with some enzyme and released into cytoplasm. So, they may also act directly to PKC like DG released from phosphoinositide by PLC.

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References

1. Murakami-Murofushi,K., Shioda,M., Kaji,K., Yoshida,S., and Murofushi,H. (1992) Inhibition of eukaryotic DNA polymerase with a novel lysophosphatidic acid (PHYLPA) isolated from myxoamoebae of *Physarum polycephalum*. J.Biol.Chem. 267, 21512-21517
2. Takahashi,Y., Shimada,Y., Shioda,M., Yoshida,S., Muofushi,H., and Murakami-Murofushi,K. (1993) Isolation of a new species of *Physarum* lysophosphatidic acid,PHYLPA, and its effect on DNA polymerase activity. Cell Struct. Funct. 18,135-138
3. Murakami-Murofushi,K., Kobayashi,S., Onimura,K. Matsumoto,M., Shioda,M., Yoshida,S., Shoji,M., and Murofushi,H. (1995) Selective inhibition of DNA polymerase α family with chemically synthesized derivatives of PHYLPA,a *Physarum* unique lysophosphatidic acid. Biochim.Biophys.Acta 1258, 57-60
4. Murakami-Murofushi,K., Kaji,K., Kano,K., Fukuda,M., Shioda,M., and Murofushi,H. (1993) Inhibition of cell proliferation by a unique lysophosphatidic acid, PHYLPA, isolated from *Physarum polycephalum*: Signaling events of antiproliferative action by PHYLPA. Cell Struct. Funct. 18, 363-370
5. Tigyi,G., Dyer,D., Matute,C., and Miledi,R. (1990) A serum factor that activates the phosphatidylinositol signaling system in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 87, 1521-1525
6. Tigyi,G., Henschen,A., and Miledi,R. (1991) A factor that activates oscillatory chloride currents in *Xenopus* oocytes copurified with a subfraction of serum albumin. J.Biol.Chem. 266, 20602-20609
7. Tigyi,G., and Miledi,R. (1992) Lysophosphatidates bound to serum albumin activate membrane currents in *Xenopus* oocytes and neurite retraction in PC12 pheochromocytoma cells. J. Biol. Chem. 267,21360-21367
8. Moolenaar,W.H., Jalink,K., and van Corven,E. (1992) Lysophosphatidic acid: a bioactive phospholipid with growth factor-like properties. Rev. Physiol. Biochem. Pharmacol. 119, 47-65
9. Jalink,K., van Corven,EJ., and Moolenaar,W.H. (1990) Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. J. Biol. Chem. 265,12232-12239
10. Krabak,M.J., and Hui,S.W. (1991) The mitogenic activities of phosphatidate are acyl-chain-length dependent and calcium independent in C3H/10T1/2 cells. Cell Regul. 2, 57-64
11. van Corven,EJ., Groenink,A., Jalink,K., Eicholtz,T., and Moolenaar,W.H. (1989) Lysophosphatidate-induced cell proliferation: Identification and dissection of signaling pathways mediated by G proteins. Cell 59, 45-54
12. Plevin,R., MacNulty,E.E., Palmer,S., and Wakelam,M.J.O. (1991) Differences in the regulation of endothelin-1- and lysophosphatidic-acid-stimulated Ins(1,4,5)P_3 formation in rat-1 fibroblasts. Biochem. J. 280,609-615
13. van Corven,EJ., Rijswik,A., Jalink,K., van der Bend,R.L., van Blitterswijk,W.J., and Moolenaar, W.H. (1992) Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin. Biochem. J. 281, 163-169
14. van Corven,EJ., Hordijk,P.L., Medema,R.H., Bos,J.L., and Moolenaar,W.H. (1993) Pertussis toxin-sensitive activation of p21ras by G protein-coupled receptor agonists in fibroblasts. Proc. Natl. Acad. Sci. USA 90, 1257-1261
15. Gerrard,J.M., and Robinson,P. (1989) Identification of the molecular species of lysophosphatidic acid

produced when platelets are stimulated by thrombin. Biochim Biophys. Acta 1001, 282-285
16.Eicholtz,T. Jalink,K., Fahrenfort,I., and Moolenaar,W.H. (1993) Biochem. J. 291, 677-680
17.Tokumura,A., Harada,K., Fukuzawa,K., and Tsukatani,H. (1986) Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. Biochim. Biophys. Acta 875, 31-38
18.Fukami,K., and Takenawa,T. (1992) Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potential mitogenic signal. J. Biol. Chem. 267, 10988-10993
19.Yasuda,I., Kishimoto,A., Tanaka,S., Tominaga,M., Sakurai,A. and Nishizuka,Y. (1990) A synthetic peptide substrate for selective assay of protein kinase C. Biochem. Biophys. Res. Commun. 166, 1220-1227
20.Kobayashi,S., Tokunoh,R., Shibasaki,M., Shinagawa,R., and Murakami-Murofushi,K. (1993) Synthesis of 1-*O*-acylglycerol 2,3-cyclic phosphate:Determination of the absolute structure of PHYLPA, a specific inhibitor of DNA polymerase. Tetrahedron Lett. 34, 4047-4050
21.Tilley,B.C., van Parden,P.A., Vwekan,I., Wirtz,K.W., De Lant,S.W., and Moolenaar,W.H. (1987) Inositol phosphate metabolism in bradykinin-stimulated human A431 carcinoma cells. Relationship to calcium signaling. Biochem. J. 244, 129-135
22.Konnno,Y., Ohno,S.,Akita,Y., Kawasaki,H., and Suzuki,K., (1989) J. Biochem.(Tokyo) 106, 673-678
23.Ohno,S., Akita,Y., Hata,A., Osada,S., Kubo,K., Konno,Y., Akimoto,K., Mizuno,K., Saido,T., Kuroki,T., and Suzuki,K. (1991) Structural and functional diversities of a family of signal transducing protein kinases, protein kinase C family; two distinct classes of PKC, conventional cPKC and novel nPKC. Adv. Enzyme Regul. 31, 287-303
24.Ohno,S., Akita,Y., Konnno,Y., Imajoh,S., and Suzuki,K. (1988) A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. Cell 53, 731-741
25.Ono,Y., Fujita,T., Ogita,K., Kikkawa,U., Igarashi,K., and Nishizuka,Y. (1988) The Structure, expression, and properties of additional members of the protein kinase C family. J. Biol. Chem 263, 6927-6932
26.Osada,S., Mizuno,K., Saido,T.C., Akita,Y., Suzuki,K., Kuroki,T., and Ohno,S. (1990) A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. J. Biol. Chem. 265, 22434-22432.
27. Matsuno,M., Kaji,K., Utakoji,T., and Hosoda,K. (1982) J. Gerontol. 37, 33-37
28.O'Farrell,P.H. (1975) High resolution two-demensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021
29.Rodriquez-Pena,A.,and Rozengurt,E. (1986) Phosphorylation of acidic Mr 80,000 cellular protein in a cell-free system and intact Swiss 3T3 cells:a specific marker of protein kinase C acticity. EMBO J. 5, 77-83
30.Nishizuka,Y. (1984)The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308, 693-698
31.Nishizuka,Y. (1986) Studies and perspectives of protein kinase C. Science 233, 305-312
32.Nishizuka,Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334,661-665
33.Liliom.K., Murakami-Murofushi,K., Kobayashi,S., Murofushi,H., and Tigyi,G. (1996) *Xenopus* oocytes express multiple receptors fro LPA-like lipid mediators. Am. J. Physiol. 270, C772-C777
34.Takahashi,Y., Shimada,Y., and Murakami-Murofushi,K. (1992) Fatty acid species of bio-active lysophosphatidic acid from haploid myxoamoebae of *Physarum polycephalum.* Nat. Sci. Rep. Ochanomizu Univ. 43, 97-102