The Role of Membrane Phospholipid Hydrolysis in Cell Proliferation and Differentiation

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神戸大学大学院自然科学研究科

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The Role of Membrane Phospholipid Hydrolysis in Cell Proliferation and Differentiation

（細胞の増殖・分化における細胞膜リン脂質加水分解反応の役割）

平成５年３月

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Summary

In most cells, when extracellular signals evoke intracellular events, hydrolysis of inositol phospholipids by phospholipase C is initiated to produce inositol 1,4,5-trisphosphate which induces calcium mobilization, and 1,2-diacylglycerol which activates protein kinase C. However, this calcium signal is normally transient, while the resulting physiological responses such as cell proliferation and differentiation often persist longer. It is becoming clear that signal-induced hydrolysis of other membrane phospholipids, particularly choline phospholipid, by phospholipase D and phospholipase A_2 also takes part in cell signaling for long-term cellular responses such as proliferation and differentiation. The products of hydrolysis of these phospholipids may enhance and prolong the activation of protein kinase C and then induce such physiological responses. The present studies were undertaken to investigate the potential roles of the products of the reaction hydrolyzed by phospholipase A_2 in evoking long-term cellular responses by the analysis of T lymphocyte activation as a model system, and also to explore effects of the metabolite of the reaction hydrolyzed by phospholipase A_2 on the activity of protein kinase C, which is a possible target for the metabolite.

In Chapter 1, it will be shown that, when phospholipase A_2 is added exogenously, the activation of human resting T lymphocytes that is induced by a membrane-permeant diacylglycerol plus calcium ionophore is greatly potentiated, as determined by the expression of α-subunit of the interleukin-2 receptor and by the amount of DNA synthesis. The effect of phospholipase A_2 on this potentiation can be reproduced by lysophosphatidylcholine, one of the products of phosphatidylcholine hydrolysis by this enzyme. Lysophosphatidylcholine *per se* is inactive unless both of the membrane-permeant diacylglycerol and Ca^{2+}-ionophore are present. *cis*-Unsaturated fatty acids, the other products of phosphatidylcholine hydrolysis by phospholipase A_2,

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are ineffective for T lymphocyte activation, both in the presence and absence of the membrane-permeant diacylglycerol and Ca^{2+}-ionophore. This effect of lysophosphatidylcholine is also observed when the diacylglycerol is replaced by a tumor-promoting phorbol ester, which is similar to 1,2-diacylglycerol in molecular structure, and mimics diacylglycerol to activate protein kinase C. Other lysophospholipids including lysophosphatidylserine, lysophosphatidylinositol, and lysophosphatidic acid are inert, except for lysophosphatidylethanolamine which is far less effective than lysophosphatidylcholine. Tracer experiments with radioactive choline have indicated that, when T lymphocytes are stimulated with an antigenic signal, lysophosphatidylcholine is indeed produced in a time dependent fashion. It is suggested that lysophosphatidylcholine is involved in the signal transduction pathway through protein kinase C, eventually leading to long-term cellular responses.

In Chapter 2, to examine whether protein kinase C is one of the targets of the lysophosphatidylcholine action, the effect of lysophosphatidylcholine on purified protein kinase C subspecies is investigated. The activities of the α-, β-, and γ-subspecies will be shown herein to be enhanced by lysophosphatidylcholine at low concentrations and suppressed at high concentrations, when assayed in the presence of phosphatidylserine, diacylglycerol, and physiologically low concentrations of Ca^{2+} with H1 histone as a phosphate acceptor protein. The activities of the δ- and ε-subspecies are not enhanced by the lysophospholipid. Kinetic analysis indicates that lysophosphatidylcholine increases the affinity of the α-, β-, and γ-subspecies for phosphatidylserine, but does not replace phosphatidylserine for the activation of the enzyme family. Lysophosphatidylcholine does not appear to affect the sensitivity of the enzyme to diacylglycerol. Among various lysophospholipids tested, lysophosphatidylcholine is most effective. Lysophosphatidylinositol, lysophosphatidylserine, and lysophosphatidic acid are less effective, whereas lysophosphatidylethanolamine is barely effective. Comparison of several molecular species of

lysophosphatidylcholine with different acyl moieties indicates that palmitoyl-lysophosphatidylcholine is most effective for the enhancement of the activity of protein kinase C. The enhancement of protein kinase C activity by lysophosphatidylcholine is also observed with other phosphate acceptor proteins such as myelin basic protein and myristoylated alanine-rich C kinase substrate. It is plausible that lysophosphatidylcholine, a product of the signal-induced membrane-phospholipid hydrolysis, may directly enhance the activity of several protein kinase C subspecies, and thereby induce long-term cellular responses such as proliferation and differentiation.

Abbreviations

aPKC: atypical protein kinase C.
p-APMSF: (*p*-amidinophenyl)methanesulfonyl fluoride.
cAMP: cyclic AMP.
CBB: Coomassie brilliant blue.
CD3: cluster of differentiation 3.
CD4: cluster of differentiation 4.
cPKC: conventional or classical protein kinase C
DG: diacylglycerol.
DiC_8: dioctanoylglycerol.
DMSO: dimethyl sulfoxide.
IL-1: interleukin-1.
IL-2: interleukin-2.
IL-2Rα: α subunit of the interleukin 2 receptor.
IP_3: inositol 1,4,5-trisphosphate.
lysoPA: lysophosphatidic acid.
lysoPC: lysophosphatidylcholine.
lysoPE: lysophosphatidylethanolamine.
lysoPI: lysophosphatidylinositol.
lysoPS: lysophosphatidylserine.
MARCKS: myristoylated alanine-rich C kinase substrate.
MHC II: class II major histocompatibility complex.
nPKC: novel or new protein kinase C.
PBS: phosphate-buffered saline.
PC: phosphatidylcholine.
PIP_2: phosphatidylinositol 4,5-bisphosphate.
PKC: protein kinase C.
PLA_2: phospholipase A_2.
PLC: phospholipase C.
PLD: phospholipase D.
PS: phosphatidylserine.
SDS: sodium dodecyl sulfate.
SDS/PAGE: SDS-polyacrylamide gel electrophoresis.
TCR: T cell (lymphocyte) receptor.
TLC: thin-layer chromatography.
TNF: tumor necrosis factor.
TPA: 12-*O*-tetradecanoylphorbol-13-acetate.

General Introduction

1. Inositol phospholipid turnover and PKC activation in signal transduction—historical background.

The biochemical basis of the transduction of extracellular signals into intracellular events and of the subsequent cellular responses has long been a subject of great interest. Various extracellular signals such as hormones, neurotransmitters, antigens, growth factors, and other biologically active substances do not penetrate through cell membranes. Instead, these signals (first messengers) normally interact with their specific cell-surface receptors, thereby the information from the extracellular signals may be transduced into the cell to control the intracellular events through actions of various second messengers. Cyclic AMP (cAMP) was first found as a second messenger [reviewed in (1)]. In this intracellular signal transduction pathway, cAMP-dependent protein kinase plays crucial roles in the regulation of a variety of cellular functions through protein phosphorylation reactions [reviewed in (2)]. On the other hand, in another signal transduction pathway which does not utilize cAMP as a second messenger, inositol phospholipid hydrolysis in cell membranes was shown to be induced by stimulation of cell-surface receptors [reviewed in (3)]. It was later proposed that the inositol phospholipid hydrolysis is associated with an increase in the intracellular Ca^{2+}, which appears to mediate a variety of subsequent cellular responses [reviewed in (4)].

In 1983, Berridge *et al.* demonstrated that inositol 1,4,5-trisphosphate (IP_3), one of the products of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by phospholipase C (PLC), serves as a mediator of Ca^{2+} mobilization from its intracellular store in the compartment of endoplasmic reticulum [reviewed in (5)]. Nishizuka *et al.* provided evidence that 1,2-diacylglycerol (DG), the other product of PIP_2 hydrolysis by PLC, is essential for the activation of protein kinase C (PKC), which is a protein serine/threonine kinase [reviewed in

(6)]. The signal transduction pathway through this protein phosphorylation is separate from, and synergistic to, the Ca^{2+} signaling pathway. Therefore, the extracellular signals elicit PIP_2 hydrolysis by PLC to produce two second messengers, IP_3 and DG, and then both Ca^{2+} mobilization induced by IP_3 and PKC activation induced by DG act synergistically to cause subsequent various cellular responses (in a left part of Fig. 1A).

PKC activation and Ca^{2+} mobilization are indeed well known to play crucial roles in physiological responses to various hormones and neurotransmitters [reviewed in (7, 8)]. In these studies, membrane-permeant DGs or phorbol esters which activate PKC, and Ca^{2+}-ionophores which increase intracellular Ca^{2+} concentration, have been frequently used as tools to investigate the molecular mechanism of cellular responses of intact cells. Ca^{2+}-ionophores such as ionomycin, which enable Ca^{2+} to pass selectively through cell membranes, can induce Ca^{2+} influx from extracellular spaces. DGs that possess two long fatty acyl moieties are normally insoluble and cannot be intercalated into cell membranes. When one or both acyl moieties are replaced by shorter acyl chains, the resulting DGs such as dioctanoylglycerol (DiC_8) obtain detergent-like properties and can be easily dispersed to activate PKC without damage to intact cell membranes. By using this procedure, the pivotal role of PKC in signal transduction was first demonstrated in the release of serotonin from platelets (9). Furthermore, it was noticed that phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent-tumor promoter, mimic the action of DG, activate PKC directly (10), and synergize with Ca^{2+}-ionophore to induce various physiological cellular responses. Phorbol esters have stronger actions than DG for cellular responses, since they are metabolically stable comparing with DG. Phorbol esters have a structure similar to the glycerol backbone and the acyl chains of membrane-permeant DG. The present studies described below employed ionomycin as a Ca^{2+}-ionophore, DiC_8 as a

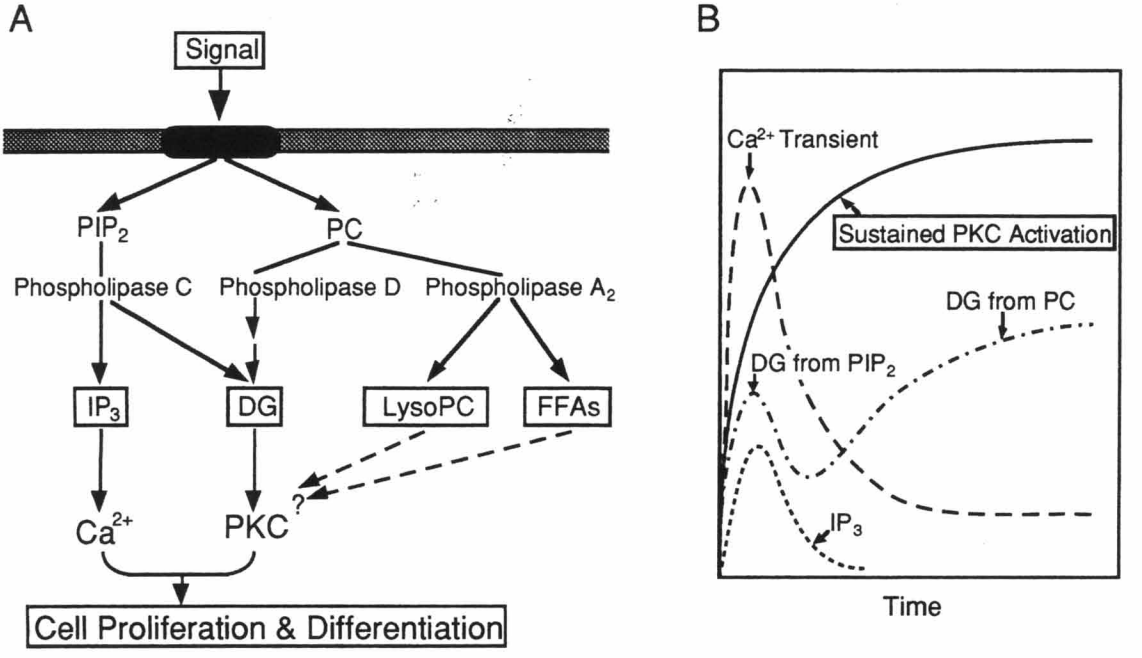

Fig. 1. Schematic representation of agonist-induced membrane phospholipid degradation and the time course of metabolism.

A, The agonist-induced membrane phospholipid degradation for cell proliferation and differentiation. Details of the pathway of DG formation from PC hydrolysis by phospholipase D are described in the text. FFAs, *cis*-unsaturated (free) fatty acids. B, Presumptive time course of metabolism of various signaling molecules derived from phospholipids. Details are described in the text. [This figure is adapted from review (11)].

membrane-permeant DG, and TPA as a phorbol ester, unless otherwise specified.

2. Sustained DG elevation and PKC activation for cell proliferation and differentiation.

DG formation, as a result of the hydrolysis of PIP_2 as described above, is normally transient, and temporally corresponds to the formation of IP_3 (Fig. 1B). The transient PKC activation by DG from PIP_2 hydrolysis may be sufficient for short-term cellular responses such as secretion and muscle contraction. However, it seems insufficient to cause long-term cellular responses such as cell proliferation and differentiation. It is conceivable that other mechanisms may also be involved in long-term cellular responses [reviewed in (11-13)]. Several lines of evidence have recently indicated that a sustained phase of DG elevation is frequently observed after the transient DG formation derived from PIP_2 hydrolysis. This second phase of DG formation is most likely due to the hydrolysis of phosphatidylcholine (PC) in various stimulated cells [reviewed in (14, 15), Fig. 1B]. This conclusion comes from extensive analysis of the fatty acid composition of the DG. As PC is the most abundant phospholipid (about 50%) in cell membranes, this phospholipid may be a major source of DG at relatively later phase of cellular responses.

Tracer experiments indicate that the metabolic turnover rate of DG is extremely rapid (16). On the other hand, phorbol esters that mimic DG for PKC activation are metabolically stable, and thus the cellular responses caused by phorbol esters could differ from those caused by a membrane-permeant DG. Recent experiments with repeated additions of a membrane-permeant DG have confirmed that the sustained activation of PKC is prerequisite for causing long-term cellular responses, such as activation of T lymphocytes (16, 17) and differentiation of human promyelocytic leukemia cells (HL-60) to macrophages (18). On the other hand, only a single dose of phorbol ester can cause similar

cellular responses. Therefore, it is possible to conclude that prolonged PKC activation evoked by sustained, second phase of DG formation from PC is necessary for the long-term cellular responses such as cell proliferation and differentiation.

3. Sustained PKC activation and phospholipases.

Many types of phospholipases including phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD) are responsible for the signal-induced phospholipid degradation [reviewed in (19), Fig. 1A]. Phosphoinositide-specific PLC produces DG, which appears in a first phase. Several mechanisms may be responsible for the signal-induced formation of DG from PC in the second phase. PC-specific PLC, which produces DG and phosphocholine from PC directly, has been proposed to occur in several tissues [reviewed in (14, 15)]. However, the relationship between this type of PLC and the signal-induced generation of DG remains unclear. It is more likely that PC is hydrolyzed by PLD in a signal dependent manner, resulting in the formation of phosphatidic acid, which is then converted to DG by the removal of its phosphate [reviewed in (14, 15), Fig. 1A]. It is proposed that sustained DG formation by PLD is responsible for prolonged PKC activation [reviewed in (11-15), Fig. 1A].

PLA_2 hydrolyzes PC to liberate *cis*-unsaturated fatty acids and lysophosphatidylcholine (lysoPC, Fig. 1A). This enzyme is ubiquitous in mammalian tissues, and it has been proposed that it may also be activated by receptor stimulation (20, 21). Therefore, great attention has been attracted to the role of PLA_2 in various physiological responses and probably the sustained activation of PKC as discussed below. PLA_2 consists of at least three groups in mammalian tissues [reviewed in (22, 23)]. Group I is a digestive enzyme derived from pancreas. Group II is found frequently in exudates at various sites of inflammation. Group I and II are secretory forms to extracellular spaces. On the other hand, it has been reported that cytosolic PLA_2 shows several distinct

properties such as the specificity of fatty acid moieties of phospholipids, Ca^{2+}-sensitivity of its enzymatic activity, and amino acid sequence (24-26). This group of cytosolic PLA_2 is activated by some growth factors, and this activation is long-lasting (27). Therefore, it is likely that PLA_2 produces some second messengers, and then evokes sustained PKC activation, resulting in the induction of long-term cellular responses such as cell proliferation and differentiation. It is possible, however, that both secretory group I and group II PLA_2 are also involved in the long-term cellular responses.

In cell-free enzymatic systems, *cis*-unsaturated fatty acids, which are the products of PC hydrolysis by PLA_2, are reported to activate PKC directly (28, 29), and also to enhance DG-dependent activation of PKC (30-32). Furthermore, several *cis*-unsaturated fatty acids, such as oleic, linoleic, and linolenic acids, when added exogenously to cells, greatly potentiate short-term cellular responses such as platelet activation to cause serotonin release (33). However, it remains unclear whether PLA_2 and its metabolites are involved in the sustained PKC activation and long-term cellular responses such as cell proliferation and differentiation. In the present studies, it will be investigated to examine the role of PLA_2 and lysoPC, which is the other product of PC hydrolysis by PLA_2, in the long-term cellular responses, with analysis of T lymphocyte activation as a model system. In the second part of the present studies, it will be investigated to see whether lysoPC may take part directly in PKC activation in a cell-free enzymatic system.

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Chapter 1

Role of Phospholipase A_2 in Cell Proliferation and Differentiation

I. Introduction

I-1. T lymphocyte activation as a model of cell proliferation and differentiation.

When foreign proteins such as virus particles invade into animal body, the first step in immune responses is phagocytosis of the proteins by antigen-presenting cells such as macrophages. The engulfed antigen proteins are digested to peptide fragments within the cells (antigen processing), and then the fragments are presented on class II major histocompatibility complex (MHC II) at their cell surface (antigen presentation). In the next step, the antigen-presenting cells bind to one of the helper T lymphocyte clones, which selectively recognizes the specific antigen, among more than 1×10^7 clones. The interaction between these distinct cells is mediated by two kinds of protein–protein interaction. First, the antigen on MHC II is recognized by the T cell (lymphocyte) receptor (TCR, Fig. 2). The TCR has immunoglobulin-like domains which recognize the specific antigen. One T lymphocyte clone expresses only one kind of TCR, namely one T lymphocyte clone recognizes only one kind of antigen. The second interaction is mediated with CD4 (cluster of differentiation 4, which is expressed selectively on helper T lymphocyte)–MHC II complex (Fig. 2). This binding ensures that T lymphocytes do not respond to free antigen nor unoccupied MHC II. These two interactions and interleukin-1 (IL-1), which is secreted from the antigen-presenting cell, induce the T lymphocyte to proliferate and differentiate into the cell possessing the ability that induces the proliferation of B lymphocytes and

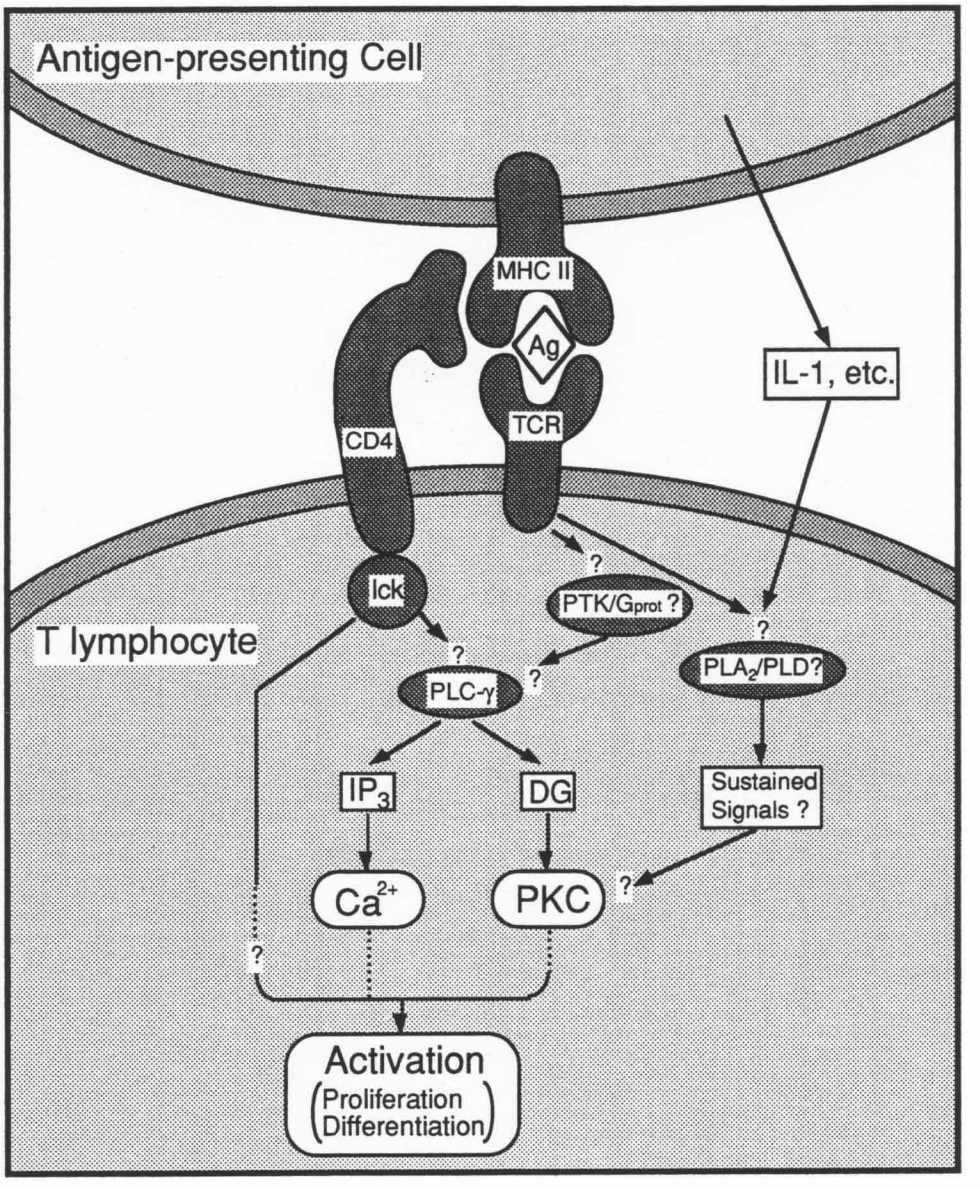

Fig. 2. Signaling events initiated by antigen-dependent T lymphocyte receptor activation.

The signal transduction pathways for helper T lymphocyte activation are schematically represented. MHC II, class II major histocompatibility complex; Ag, antigen; CD4, cluster of differentiation 4; TCR, T cell (lymphocyte) receptor; IL-1, interleukin-1; lck, $p56^{lck}$ protein tyrosine kinase; PTK, unidentified protein tyrosine kinase; G_{prot}, unidentified GTP-binding protein; PLC-γ, the γ-subspecies of PLC. For other abbreviations and details, refer the text.

other cells. This induction of the proliferation and differentiation is called T lymphocyte activation. The activated T lymphocytes promote B lymphocytes, which produce the immunoglobulin which recognizes a specific antigen selectively, to proliferate through actions of interleukins, resulting in the activation of immune responses.

A pivotal event in generation of the immune responses to most antigens is the clonal activation of antigen-specific T lymphocytes. From extensive biochemical and immunological analysis, the mechanisms of T lymphocyte activation are becoming clear [reviewed in (1, 2), Fig. 2]. The presented antigen on MHC II is recognized by TCR on a resting helper T lymphocyte, and the MHC II is recognized by CD4 at the same time. Consequently, $p56^{lck}$, a protein tyrosine kinase that is associated with CD4, is activated. It is conceivable that this tyrosine kinase phosphorylates PLC γ-subspecies to induce the activation of this phospholipase (3-5). It has been also proposed that the antigen-stimulated TCR may induce PLC activation through action of either an unidentified protein tyrosine kinase ($p59^{fyn}$?) or an unidentified GTP-binding protein [reviewed in (1, 2)]. The activated PLC hydrolyzes PIP_2 to produce IP_3 and DG, which induce Ca^{2+} mobilization and PKC activation, respectively. However, these two signals are not sufficient for the activation of T lymphocytes, resulting in the induction of immune tolerance. The full activation of T lymphocytes normally requires so-called accessory signals such as IL-1 (Fig. 2). It is reported that IL-1 elevates DG production derived from PC but not from PIP_2 (6). Furthermore, IL-1 also seems to induce the activation of PLA_2 (7). Thus, it is possible that IL-1 is involved in the sustained PKC activation through the activation of PLD and/or PLA_2. The lck protein tyrosine kinase may be involved in T lymphocyte activation through another signal transduction pathway (8). These signals are transduced to the nucleus and induce gene expression of more than 100 genes including interleukin-2 (IL-2) and the α subunit of IL-2 receptor (IL-2Rα) (9, 10). IL-2 secreted from the helper T lymphocytes induces proliferation of other classes of

T lymphocytes such as cytotoxic T lymphocytes, and the activated helper T lymphocytes leads to their own proliferation, through interaction of IL-2Rα with IL-2 secreted by themselves as an autocrine mechanism.

T lymphocyte activation has been used as a model system for the cell proliferation and differentiation (11, 12). T lymphocyte activation has many advantages for the analysis of cell proliferation and differentiation: 1, Most of T lymphocytes prepared from peripheral venous blood are in a resting state, G_0 phase of the cell cycle. Thus, this cell is suitable for the analysis of progression of cell cycle and signal transduction pathway after the stimulation by mitogen. 2, Since various surface antigens such as IL-2Rα are well characterized as differentiation markers, the extent and progression of the differentiation are able to be easily determined using fluorescent anti-surface antigen monoclonal antibodies by flow cytometry. Analysis of the cell proliferation can be also easily made by measuring the [^{3}H]thymidine incorporation to DNA. 3, As described above, various studies on the signal transduction pathways in T lymphocyte have been made extensively, and many knowledges of biochemical, cytological, and immunological aspects have already accumulated. 4, The highly purified resting T lymphocytes are easily prepared from peripheral venous blood of healthy volunteers, and various cell lines such as Jurkat cell line which responds to antigenic signals are also available. Therefore, in this study, investigation of the role of sustained PKC activation and PLA_2 in the cell proliferation and differentiation was performed by analysis of T lymphocyte activation as a model system.

I-2. T lymphocyte activation and sustained PKC activation.

It has been well known that T lymphocyte activation requires both PKC activation by phorbol esters and Ca^{2+} mobilization [reviewed in (13, 14)]. However, the addition of a membrane-permeant DG in the presence of Ca^{2+}-ionophore is insufficient to induce T lymphocyte activation (15), although DiC_8 is

able to induce short-term cellular responses such as platelet activation. The phorbol esters are metabolically stable. On the other hand, radioactive tracer experiments have shown that DiC_8 is rapidly metabolized by various enzymes such as DG kinase, DG lipase, and non-specific esterase (16). Therefore, the duration of the cells exposed to DiC_8 may be a function of the amount of DiC_8 added, the frequencies of its additions, as well as the cell density in the medium (16). In fact, the number of multiple and repeated additions of DiC_8 is proportional to the induction of T lymphocyte activation. In addition, the density of T lymphocytes in the medium is inversely related to the extent of this cell activation (16). Thus, it is likely that sustained PKC activation is needed for this long-term cellular response (17). As described above, since IL-1 induces DG production by PC hydrolysis (6), it is possible that this DG formation may cause sustained PKC activation to induce T lymphocyte activation. On the other hand, since IL-1 also activates intracellular PLA_2 (7), it is equally possible that this phospholipase and its metabolites may play roles in the sustained PKC activation. The secretory group II PLA_2 may be involved in T lymphocyte activation, since this group enzyme is found frequently in exudates at various sites of inflammation. In this chapter, it will be described that PLA_2 as well as lysoPC, one of the products of PC hydrolysis by PLA_2, is involved in T lymphocyte activation, probably through the enhancement of sustained PKC activation.

II. Materials & Methods

II-1. Materials and chemicals.

Radioactive 1,2-DiC_8 (racemic 1,2-di[1-^{14}C]octanoylglycerol; 27 mCi/mmol; 1 Ci = 37 GBq), L-palmitoyllysoPC (L-[1-^{14}C]palmitoyl-lysophosphatidylcholine; 56 mCi/mol), and choline ([*methyl*-^{3}H]choline chloride; 75 Ci/mmol) were obtained from Amersham. [*methyl*-^{3}H]Thymidine (2.0 Ci/mmol) was purchased from New England Nuclear.

1,2-DiC_8 was a product of Nacalai Tesque (Kyoto). This preparation consists of a mixture of 95% DL-1,2-DiC_8 and 5% 1,3-DiC_8, as estimated by thin-layer chromatography (TLC). The three isomers (D-1,2-, L-1,2-, and 1,3-DiC_8) were rapidly isomerized to an equilibrium state nonenzymatically in aqueous medium, but 1,2-DiC_8 could be stored for at least one month at –20°C in chloroform without measurable isomerization. However, the DiC_8 employed for the present studies was a mixture of D-1,2-DiC_8 and L-1,2-DiC_8.

L-α-DecanoyllysoPC, L-α-myristoyllysoPC, L-α-palmitoyllysoPC, L-α-stearoyllysoPC, L-α-oleoyllysophosphatidic acid (lysoPA), L-α-palmitoyl-lysophosphatidylethanolamine (lysoPE), L-α-lysophosphatidyl-L-serine (lysoPS, from bovine brain), L-α-lysophosphatidylinositol (lysoPI, from soy bean), and bee venom PLA_2 were obtained from Sigma. TPA and ionomycin were purchased from LC Services (Woburn, MA) and Calbiochem-Behring, respectively. The acetoxymethyl ester of fura-2 was obtained from Dojindo Laboratories (Kumamoto). Other chemicals were purchased from commercial sources.

DiC_8 was dissolved in chloroform, and lysophospholipids were dissolved in a mixture of chloroform and methanol (1:1, vol/vol), respectively. TPA was dissolved in 100% (vol/vol) dimethyl sulfoxide (DMSO). In use, this stock solution was diluted appropriately to give a final concentration of < 0.05% DMSO, not to damage T lymphocyte. Ionomycin was dissolved in 100% (vol/vol) ethanol. In use, this stock solution was diluted appropriately to give a final

concentration of < 0.05% ethanol, not to damage T lymphocyte. These reagents were stored at –20°C in dark until use. Bee venom PLA_2 was dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and stored at 4°C.

II-2. Preparation of human peripheral resting T lymphocytes.

Human peripheral resting T lymphocytes were prepared as described (16), with slight modifications. Human peripheral venous blood was collected from healthy volunteers, into a transfusion bag containing citrate phosphate dextrose (CPD solution) as an anticoagulant. The whole blood (200 ml) is diluted with about 100 ml of PBS, and then 22.5 ml of the diluted blood samples were carefully layered onto 7.5 ml of Ficoll-Paque (Pharmacia-LKB) in sterile plastic culture tubes. Mononuclear cells (containing monocytes and lymphocytes) were separated by a density gradient centrifugation (400 × g, 30 min at room temperature). After the upper layer (containing plasma and platelet) was removed, the intermediate layer was collected to sterile culture tubes by aspiration. Washing the mononuclear cells was performed twice to remove contaminating platelets as below. Mononuclear cells fraction was diluted with PBS, and a centrifugation (100 × g, 20 min at room temperature) was performed. The precipitate was suspended in PBS. After washing the mononuclear cells, the cells were suspended in RPMI 1640 medium (Flow Laboratories), and then incubated for 1 hr at 37°C in a culture dish in humidified 5% CO_2/95% air, in order to allow monocytes to adhere on the plastic dish. The nonadherent cells (containing lymphocytes) were gently poured from the dish, and the residual monocytes were removed again as described above. The nonadherent cells were passed through a 50-ml disposable syringe stuffed about 15 cm^3 (5.3 g) of sterile nylon-wool to remove accessory cells containing B lymphocytes, as described (18). The accessory cell-depleted preparation obtained by this method contained 95% T lymphocyte judged by a flow cytometer (Cyto ACE-150;

Japan Spectroscopic, Tokyo) with a fluorescein-conjugated anti-CD3 (T lymphocyte receptor) monoclonal antibody (Immunotech S.A., France) as a T lymphocyte marker.

II-3. Assessment of T lymphocyte activation.

II-3-1. Stimulation of resting T lymphocyte.

T lymphocytes prepared as described above were seeded at a density of 5.0×10^5 cells/ml in RPMI 1640 medium containing 5% autologous serum in 24-holes microtiterplate in humidified 5% CO_2/95% air. The cells were stimulated by the addition of ionomycin, DiC_8, and lysoPC, as specified in each experiment. DiC_8 and lysophospholipid, stored in the organic solvents, were dried under a nitrogen stream, and the residue was then sonicated in water, for 3 min at 0°C, and added to the medium to stimulate the cells.

II-3-2. Determination of IL-2Rα expression.

T lymphocytes were stimulated as described above and incubated for 16 hr. The cells were collected in Eppendorf tubes and pelleted down by a centrifugation at 500 × g for 5 min at 4°C. Following procedures were performed at 0–4°C. The pellet was resuspended in PBS, at a density of 1.0×10^7 cells/ml, and treated for 30 min with a fluorescein-conjugated anti-CD25 antibody that recognizes IL-2Rα (Beckton Dickinson) at appropriate dilutions. The cells were pelleted down again, and resuspended in PBS. The fluorescence intensity was determined by analysis of 5,000 cells with the flow cytometer (Cyto ACE-150; Japan Spectroscopic, Tokyo).

II-3-3. Thymidine incorporation assay.

T lymphocytes were stimulated as described above and incubated for 30 hr. For the last 6 hr of incubation, the cells were exposed to [^{3}H]thymidine (0.5 μCi/well). The incubation was stopped by the addition of 25% trichloroacetic

acid. The acid-precipitable materials were collected on a glass filter (Whatman; GF/A), with a vacuum apparatus. The glass filter was washed with 25% (w/v) trichloroacetic acid, and put in a glass vial containing 10 ml of liquid scintillation cocktail (66.6% toluene, 33.3% Triton X-100, 2.67 g/liter 2,5-diphenyloxazole (PPO), and 0.067 g/liter 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)). The radioactivity was measured by a liquid scintillation spectrometer.

II-4. Metabolism of 1,2-DiC_8 and lysoPC.

II-4-1. Lipid extraction.

Lipid extraction was performed by the method of Bligh and Dyer (19) with slight modifications. To the culture of T lymphocytes (1 ml) labeled with a radioactive compound, 1 ml of methanol was added, and mixed by vortexing vigorously. Next, 1 ml of chloroform and 0.1 ml of 12 N HCl were added, and mixed by vortexing vigorously. After centrifugation (300 × g, 5 min), the upper, aqueous phase was removed by aspiration, and then the lower, chloroform phase (about 1 ml) was collected. A 100 µl aliquot of the chloroform phase was usually used for TLC analysis.

II-4-2. Analysis for metabolism of 1,2-DiC_8.

Metabolism of 1,2-DiC_8 was analyzed with DL-1,2-[^{14}C]DiC_8 as described (16). The radioactive 1,2-[^{14}C]DiC_8 in chloroform was dried under a nitrogen stream, and dispersed in PBS by vigorous mixing followed by sonication for 3 min at 0°C. The material was added directly to the cell suspension at a final concentration of 50 µM (2–3 × 10^5 cpm/ml). The incubation was stopped by the addition of methanol, and then lipids were extracted as described in II-4-1. Chloroform-soluble materials were applied onto TLC plate (Merck silica gel 60 plate), and then were separated with chloroform/acetone, 96:4 (vol/vol) as a solvent system. Nonradioactive 1,2-DiC_8 and 1,3-DiC_8 were run simultaneously as authentic markers. Each spot was visualized by I_2 vapor, and the radioactivity

corresponding to 1,2-DiC_8 and its metabolites was quantitated with a BAS-2000 Bioimage analyzer (Fuji Film, Tokyo).

II-4-3. Analysis for metabolism of lysoPC.

The radioactive [^{14}C] palmitoyllysoPC was dispersed in PBS by vigorous mixing and added to the cell suspension at a final concentration of 50 μM (2–3 × 10^5 cpm/ml). The incubation was stopped by the addition of methanol, and then lipids were extracted as described in II-4-1. Chloroform-soluble materials were applied onto TLC plate (Merck silica gel 60 plate), and then were separated with chloroform/methanol/water, 65:25:4 (vol/vol) as a solvent system. Nonradioactive palmitoyllysoPC, palmitic acid, and PC were run simultaneously as authentic markers. Each spot was visualized by I_2 vapor, and the radioactivity corresponding to lysoPC and its metabolites was quantitated with a BAS-2000 Bioimage analyzer (Fuji Film, Tokyo).

II-5. Measurement of intracellular Ca^{2+} concentrations.

The intracellular Ca^{2+} concentrations were measured as described by Poenie *et al.* (20) with slight modifications. To a suspension of resting T lymphocytes (5 × 10^5 cells/ml) in 10 mM Hepes-NaOH at pH 7.4 containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose (HBS buffer), a solution of 1 mM acetoxymethyl ester of fura-2 in 100% (vol/vol) DMSO was added to give a final concentration of 5 μM of this compound. Fura-2 is a fluorescent tetracarboxylate chelator that exhibits a spectral shift in excitation maxima on binding Ca^{2+} (21). Increasing free Ca^{2+} increases the fluorescence excited by 350 nm light and decreases the fluorescence from 385 nm excitation. The ratio of fluorescence excited by 350 nm to that excited by 385 nm is a measure of intracellular free Ca^{2+} concentrations. The cell suspension was incubated for 2 hr at 37°C, washed twice with HBS buffer, and resuspended in the buffer containing 5% autologous serum and 400-μM $CaCl_2$ at a density of 5 × 10^5

cells/ml. The extracellular Ca^{2+} concentration was adjusted to a level equivalent to that in RPMI 1640 culture medium containing 5% autologous serum. The cells were stimulated as described in II-3-1. The excitations were performed at 350 nm and at 380 nm, and then the emissions were collected at 500 nm, using a CAF-100 calcium analyzer (Japan Spectroscopic, Tokyo) to calculate intracellular free Ca^{2+} concentrations.

II-6. LysoPC formation in T lymphocytes.

T lymphocytes (1×10^7 cells/ml) were labeled with [^{3}H]choline (3 μCi/ml) for 24 hr as described by Slivka and Insel (22). After incubation, the cells were washed with PBS, three times, and suspended in PBS at a density of 1.0×10^7 cells/ml. The cells were stimulated with anti-CD3 (T lymphocyte receptor) monoclonal antibody (10 μg/ml) (Immunotech, Marseille, France). At the times indicated, the incubation was stopped by the addition of methanol, and then lipids were extracted as described in II-4-1. Chloroform-soluble materials were applied onto TLC plate (Merck silica gel 60 plate), and then were separated with chloroform/methanol/water, 65:25:4 (vol/vol) as a solvent system. Nonradioactive palmitoyllysoPC was run simultaneously as an authentic marker. Lipids were visualized by I_2 vapor, and the spot corresponding to lysoPC was scraped off. The silica powder containing the spot of lysoPC was transferred to 3 ml of liquid scintillation cocktail as described in II-3-3, and the radioactivity was measured by a liquid scintillation spectrometer.

III. Results

III-1. PLA_2 and T lymphocyte activation.

At a lower cell density (1×10^5 cells/ml), T lymphocyte activation was weakly induced by a single dose of 1,2-DiC_8 as a membrane-permeant DG, in the presence of ionomycin as a Ca^{2+}-ionophore (Fig. 3, PLA_2(–), DiC_8 +, Iono +), as previously reported (16). This cell activation absolutely required both 1,2-DiC_8 and Ca^{2+}-ionophore. It has been also reported that repeated additions of 1,2-DiC_8, as well as a single dose of metabolically stable phorbol esters such as TPA, greatly enhanced this cell activation in the presence of Ca^{2+}-ionophore, suggesting that sustained PKC activation is required for the cell differentiation and proliferation (16, 17). To investigate the involvement of PLA_2 in this signal transduction through sustained PKC activation, bee venom PLA_2 was directly added to T lymphocytes exogenously. The addition of the PLA_2 greatly potentiated T lymphocyte activation caused by a single dose of 1,2-DiC_8 in the presence of a Ca^{2+}-ionophore (Fig. 3), to the same level of the activation caused by repeated doses of 1,2-DiC_8. It is important to note that PLA_2 alone was practically inactive, irrespective of the presence and absence of Ca^{2+}-ionophore, and that 1,2-DiC_8 was essential for this PLA_2 action. The action of PLA_2 was dose-dependent (data not shown). Furthermore, the secretory group II PLA_2 obtained from rat platelets was shown to enhance T lymphocyte activation, which is found frequently in exudates at various sites of inflammation (23). The secretory group II PLA_2 alone was ineffective unless 1,2-DiC_8 and Ca^{2+}-ionophore were present. Thus, it is possible that PLA_2 is involved in T lymphocyte activation through sustained PKC activation.

III-2. LysoPC and T lymphocyte activation.

PC is the most abundant phospholipid in cell membranes. PC hydrolysis by PLA_2 produces two metabolites, lysoPC and *cis*-unsaturated fatty acids. The

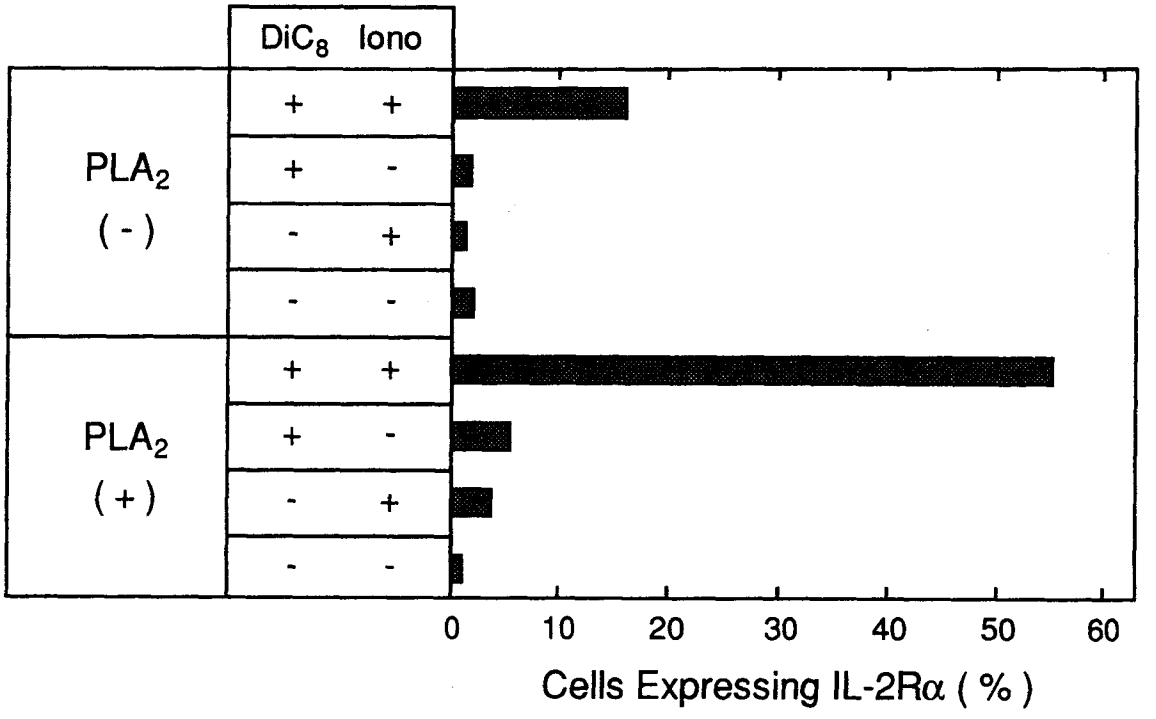

Fig. 3. Effect of bee venom PLA_2 on T lymphocyte activation induced by 1,2-DiC_8 and Ca^{2+}-ionophore.

Resting T lymphocytes (5×10^5 cells/ml) were stimulated with 1,2-DiC_8 (50 μM) and ionomycin (0.5 μM) as a Ca^{2+}-ionophore in the presence and absence of bee venom PLA_2 (10 units) as indicated. After incubation for 16 hours, the IL-2Rα expression was determined.

addition of lysoPC, instead of bee venom PLA_2, directly to the incubation medium, greatly potentiated T lymphocyte activation that was induced by a single dose of 1,2-DiC_8 plus Ca^{2+}-ionophore, as measured by IL-2Rα expression and [^{3}H]thymidine incorporation into DNA as markers of the differentiation and proliferation (Fig. 4). LysoPC *per se* was totally inactive unless both 1,2-DiC_8 and Ca^{2+}-ionophore were present as in the case of PLA_2. On the other hand, *cis*-unsaturated fatty acids, the other products of PC hydrolysis by PLA_2, did not enhance T lymphocyte activation, even in the presence of DiC_8 and Ca^{2+}-ionophore (data not shown), although these lipids greatly enhance platelet activation caused by 1,2-DiC_8 and Ca^{2+}-ionophore (24). Therefore, it is plausible that the effect of PLA_2 on T lymphocyte activation is mediated through the action of lysoPC, but not *cis*-unsaturated fatty acids.

III-3. LysoPC concentration and specificity.

The precise concentration of lysoPC that was actually effective for the potentiation of T lymphocyte activation was difficult to be measured, because the incubation medium contained serum, which traps most of lysophospholipid available for this cell activation. However, the stimulatory effect of lysoPC was evident at 10 μM, and appeared to be maximum at 100 μM, as measured by IL-2Rα expression and [^{3}H]thymidine incorporation into DNA, when the medium contained 5% autologous serum (Fig. 5). Perhaps, lysoPC was effective at <10 μM, because even at this concentration a large part of this lipids remained in the cell culture medium in association with serum proteins. At concentrations >100 μM, lysoPC was toxic and caused cell lysis.

The polar group specificity of lysophospholipid was investigated for the potentiation of T lymphocyte activation. This potentiation was specific for lysoPC, and all other lysophospholipids thus far tested were inactive under comparable conditions except for lysoPE which was 10–20% as active as lysoPC (Fig. 6). In this study, palmitoyl-lysoPC was used unless otherwise specified. Acyl group

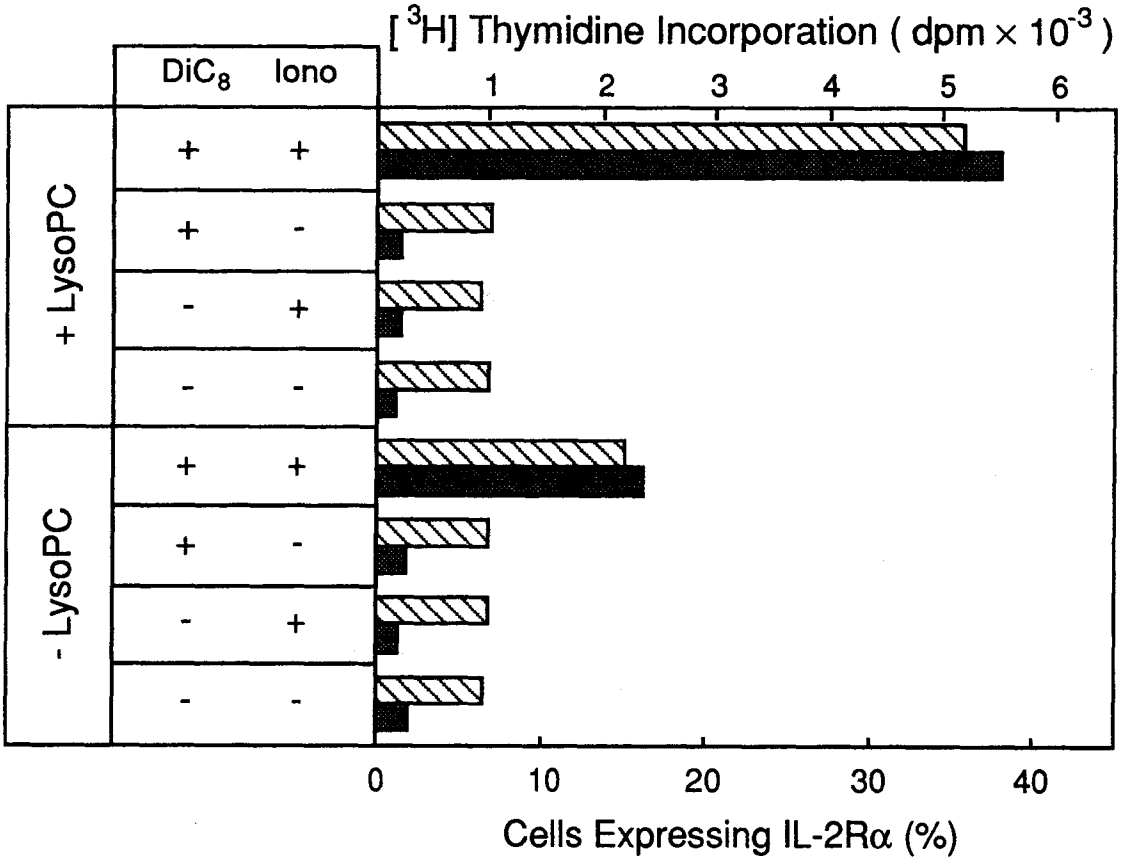

Fig. 4. Effect of lysoPC on T lymphocyte activation induced by 1,2-DiC$_8$ and Ca^{2+}-ionophore.

Resting T lymphocytes (5×10^5 cells/ml) were stimulated with 1,2-DiC$_8$ (50 μM) plus ionomycin (0.5 μM) and in the absence and presence of lysoPC (50 μM), as indicated. After incubation, the IL-2Rα expression and thymidine incorporation were determined as described in Materials & Methods. Iono, ionomycin; Hatched bars, thymidine incorporation; Shaded bars, cells expressing IL-2Rα.

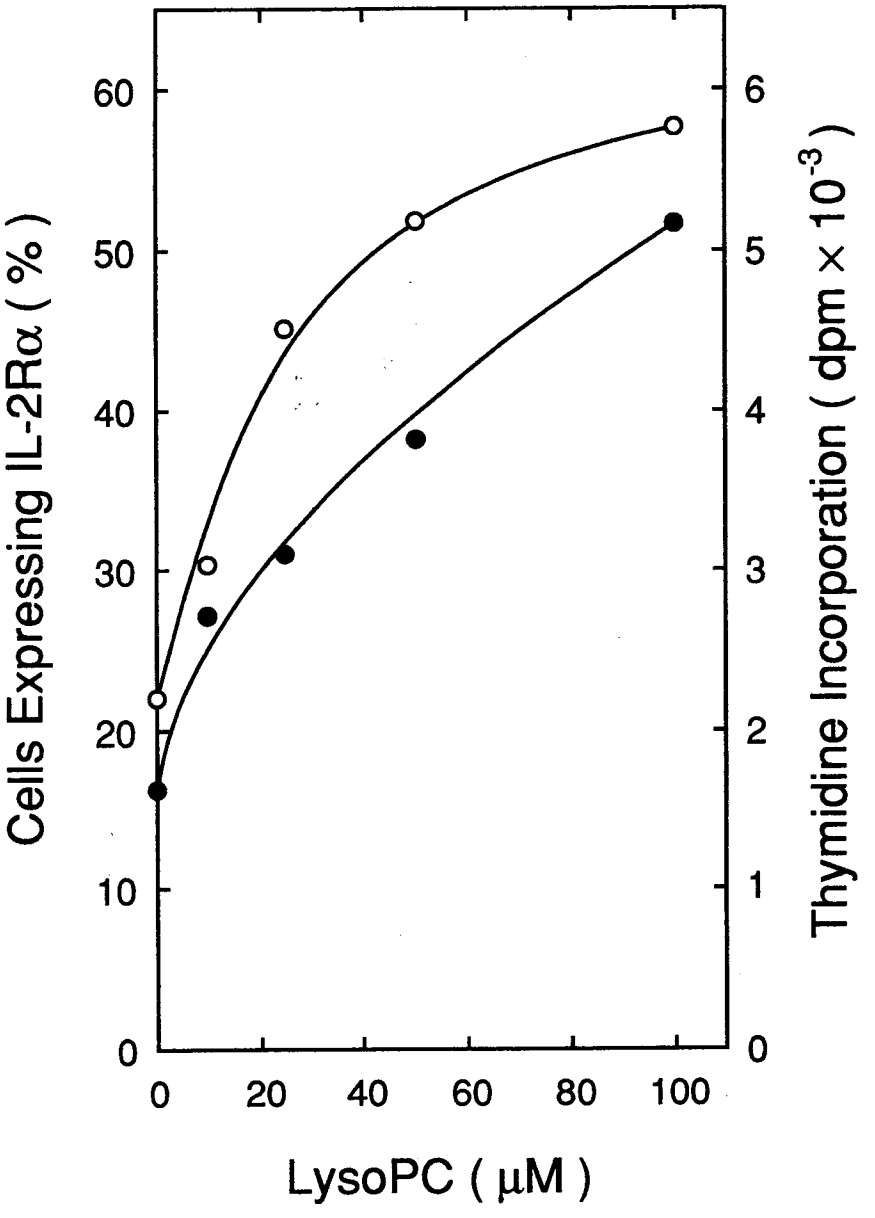

Fig. 5. Effect of lysoPC concentration on T lymphocyte activation by 1,2-DiC$_8$ and Ca^{2+}-ionophore.

Resting T lymphocytes (5×10^5 cells/ml) were stimulated with 1,2-DiC$_8$ (50 μM) plus ionomycin (0.5 μM) in the presence of various concentrations of lysoPC as indicated. IL-2Rα expression and thymidine incorporation were determined as described in Materials & Methods. ●, Cells expressing IL-2Rα; ○, Thymidine incorporation.

specificity of lysoPC was next examined for the potentiation (Fig. 6). Myristoyl (14:0, carbon chain length: number of double bond)-lysoPC, palmitoyl (16:0)-lysoPC, and stearoyl (18:0)-lysoPC were all active, whereas, decanoyl (10:0)-lysoPC was less effective for T lymphocyte activation. The acyl groups in the *sn*-1 position of PC, that are found in cell membranes, are mainly palmitoyl and stearoyl moieties (25). Thus, the potentiation of T lymphocyte activation may be induced by the lysoPC subtypes that are produced from cell membranes under physiological conditions. Since the dose dependency and specificity for lysoPC were observed, this potentiation by lysoPC may not be simply due to its non-specific toxic actions to cell membranes.

III-4. 1,2-DiC_8 and intracellular Ca^{2+} concentrations.

The activation of T lymphocytes absolutely required both 1,2-DiC_8 and Ca^{2+}-ionophore, irrespective of the presence and absence of lysoPC. LysoPC did not affect significantly the apparent half maximum concentration of 1,2-DiC_8 that was needed for this cell activation (Fig. 7A). The intracellular Ca^{2+} concentration was measured with the fura-2 procedure as described in II-5, in order to examine the role of Ca^{2+} for the potentiation of this cell activation by lysoPC. When Ca^{2+}-ionophore was added at 1 μM, the concentration of intracellular Ca^{2+} raised to about 200 nM. On the other hand, without Ca^{2+}-ionophore, the concentration is about 100 nM in both presence and absence of lysoPC. Thus, Ca^{2+}-mobilization induced by lysoPC could be neglected. It has been reported that *cis*-unsaturated fatty acids increase the Ca^{2+}-sensitivity of platelet activation that is induced by Ca^{2+}-ionophore and DiC_8 (24). However, lysoPC did not increase the sensitivity of T lymphocyte activation to Ca^{2+} (Fig. 7B). Thus, the mode of action of lysoPC for signal transduction pathway may be different from that of *cis*-unsaturated fatty acids.

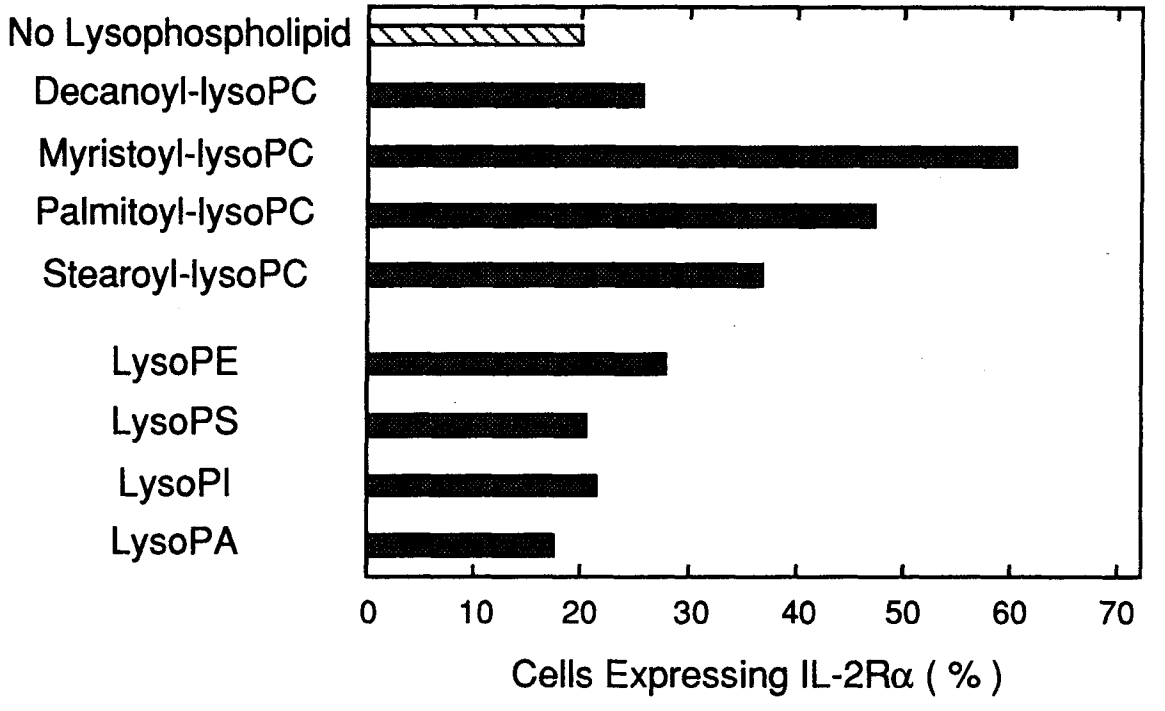

Fig. 6. Specificity of various lysophospholipids on T lymphocyte activation.

T lymphocytes (5×10^5 cells/ml) were stimulated with various lysophospholipids (50 μM) in the presence of 1,2-DiC_8 (50 μM) plus ionomycin (0.5 μM), as indicated. After incubation, the expression of IL-2Rα was measured as described in Materials & Methods.

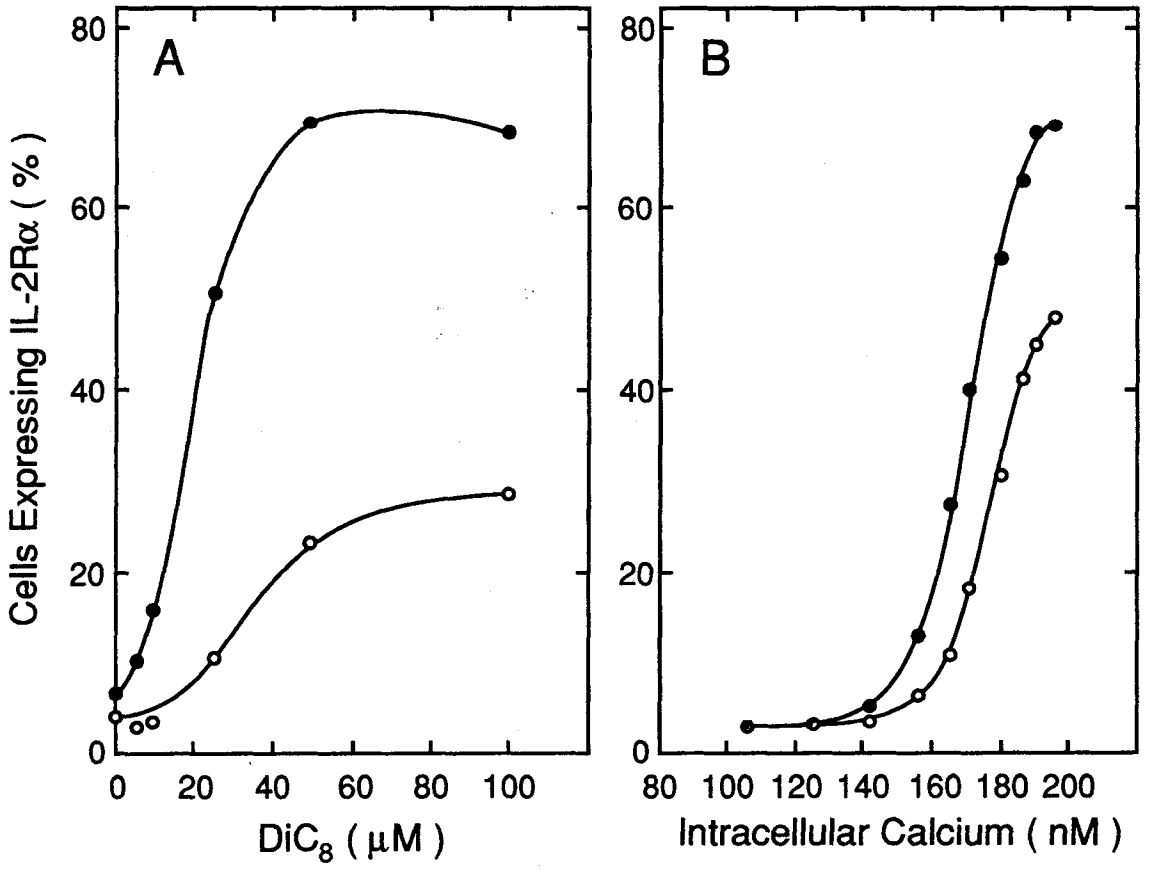

Fig. 7. IL-2Rα expression as a function of 1,2-DiC$_8$ or intracellular Ca^{2+} concentration in the presence and absence of lysoPC.

The experimental conditions were essentially identical with those given in Fig. 4, except that various amounts of 1,2-DiC$_8$ (A) or ionomycin (B) were added. ●, in the presence of lysoPC (50 μM); ○, in the absence of lysoPC. A, Effect of IL-2Rα expression on various concentrations of 1,2-DiC$_8$. B, Effect of IL-2Rα expression on various concentrations of ionomycin. Various amounts of ionomycin were added to give intracellular Ca^{2+} concentrations between 106 nM (the resting conditions) and 196 nM as indicated. The intracellular Ca^{2+} concentrations after the addition of various amounts of ionomycin were measured as described and plotted versus IL-2Rα expression.

III-5. Metabolism of lysoPC and 1,2-DiC_8.

1,2-DiC_8 added to a suspension of T lymphocytes disappeared very quickly, with the concomitant accumulation of its metabolic products (Fig. 8). This disappearance of 1,2-DiC_8 was reported to be due to its hydrolysis to produce largely octanoic acid (16). Although the detailed enzymatic basis remains unclear, it is possible that this degradation of 1,2-DiC_8 is initiated by the action of non-specific esterase rather than a stereospecific DG lipase, since the radioactive 1,2-DiC_8 is a mixture of D- and L-isomers but nevertheless disappeared rapidly. The rate of hydrolysis was not affected by the coexistence of lysoPC in the incubation medium (data not shown).

LysoPC, on the other hand, was relatively stable, and >80% of this lipid initially added still remained in the incubation medium, presumably in a form bound to serum proteins even after 6 hr (Fig. 8). This slow degradation of lysoPC is probably due to the slow delivery from serum proteins to T lymphocytes. Similar results were obtained both in the presence and the absence of 1,2-DiC_8 and Ca^{2+}-ionophore (data not shown). Palmitic acid and PC were produced gradually, as the products of the metabolism of lysoPC. Palmitic acid may be produced by the action of lysophospholipase. PC may be produced by the acylation of lysoPC. The effect of these products on T lymphocyte activation remains unclear.

Fig. 9A shows that lysoPC was no longer effective after a prolonged period of time, when 1,2-DiC_8 disappeared from the medium. In contrast, when 1,2-DiC_8 was replaced by TPA, lysoPC was always able to enhance the cell activation, presumably because TPA was metabolically stable and remained in the incubation medium (Fig. 9B). Thus, it is suggestive that lysoPC acts with DiC_8 at the same time, but does not potentiate the pre-activated cells by DiC_8 and Ca^{2+}-ionophore.

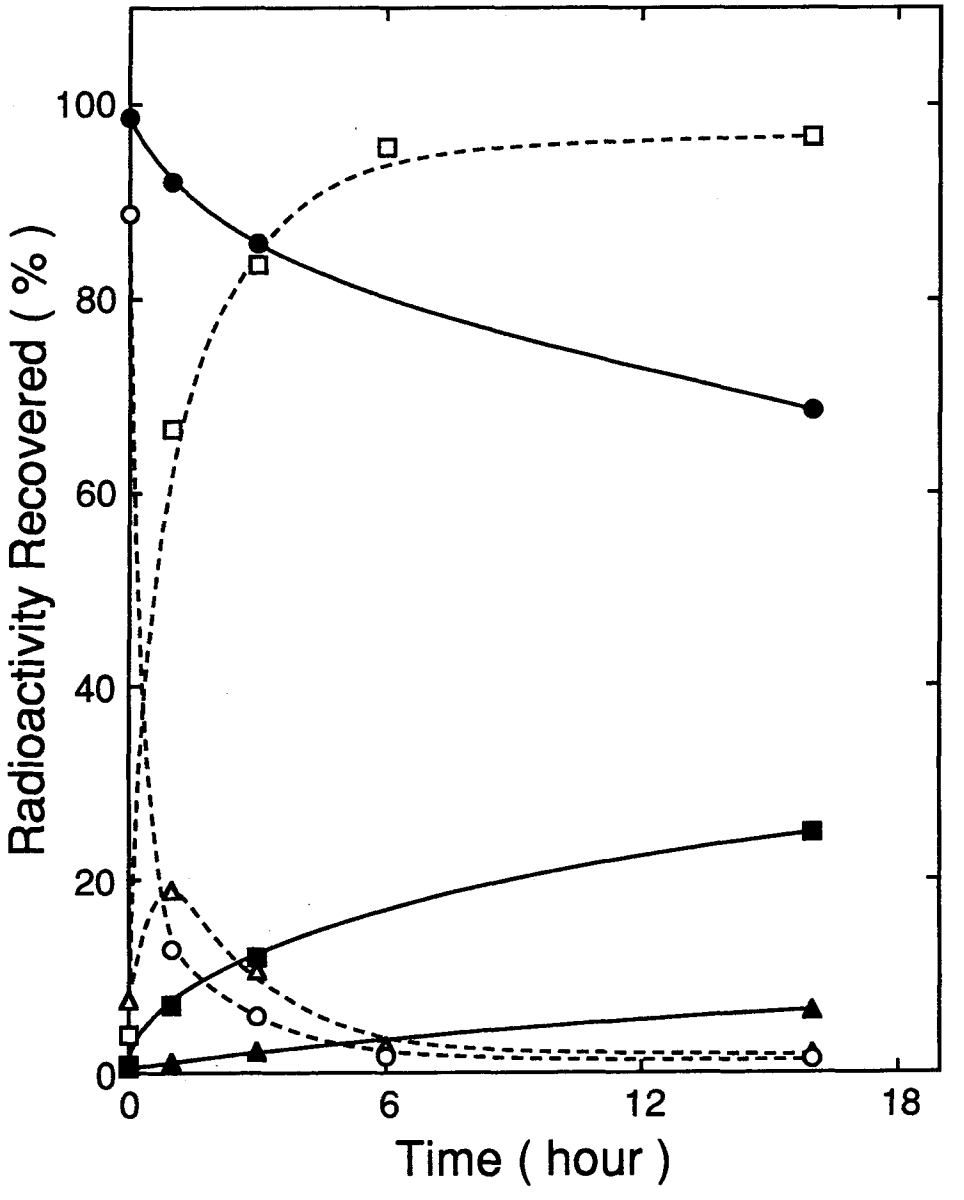

Fig. 8. Metabolism of 1,2-DiC$_8$ and lysoPC in T lymphocytes.

1,2-[^{14}C]DiC$_8$ (50 μM; 2–3 × 10^5 cpm/ml) or [^{14}C]lysoPC (50 μM; 2–3 × 10^5 cpm/ml) was added to the cell suspension (5 × 10^5 cells/ml) in RPMI 1640 supplemented with 5% autologous serum. At the various times indicated, lipids were extracted and separated by TLC. The radioactivity of each lipid was quantitated. Results are presented as percentages of the total input radioactivity added to the cell suspension. ●, lysoPC; ■, palmitic acid; ▲, PC; ○, 1,2-DiC$_8$; □, total metabolites of 1,2-DiC$_8$; △, 1,3-DiC$_8$.

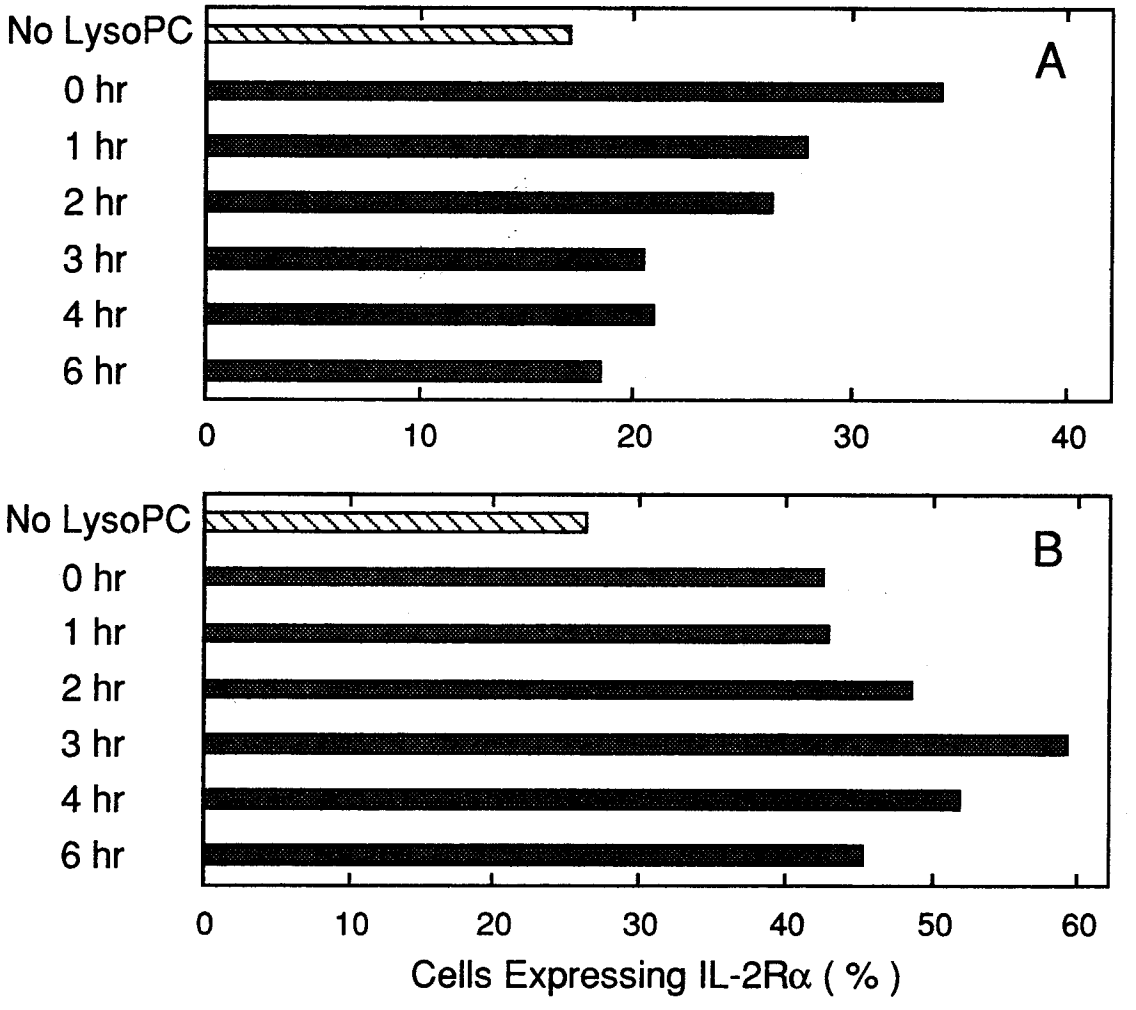

Fig. 9. Effect of delayed addition of lysoPC on IL-2Rα expression induced by 1,2-DiC$_8$ or TPA in the presence of Ca^{2+}-ionophore.

T lymphocytes were stimulated with 1,2-DiC$_8$ or TPA in the presence of ionomycin. LysoPC (50 μM) was added at the time indicated after the addition of ionomycin plus either 1,2-DiC$_8$ or TPA. After incubation for a total of 16 hr, the expression of IL-2Rα was quantitated. "No LysoPC" represents the IL-2Rα expression induced by ionomycin plus either 1,2-DiC$_8$ or TPA without lysoPC. A, Stimulation with 1,2-DiC$_8$ (50 μM) and ionomycin (0.5 μM). B, Stimulation with TPA (1 nM) and ionomycin (0.5 μM).

III-6. LysoPC formation by physiological signal.

It has been well documented that antigenic signals cause inositol phospholipid hydrolysis to produce DG (14). A preliminary experiment with radioactive choline as a tracer showed that, after T lymphocytes were stimulated with an anti-CD3 antibody as an antigenic signal, lysoPC gradually increased significantly over 1 hr (Fig. 10). Thus, it is possible that the extracellular signals such as the antigenic signal may induce the sustained accumulation of lysoPC.

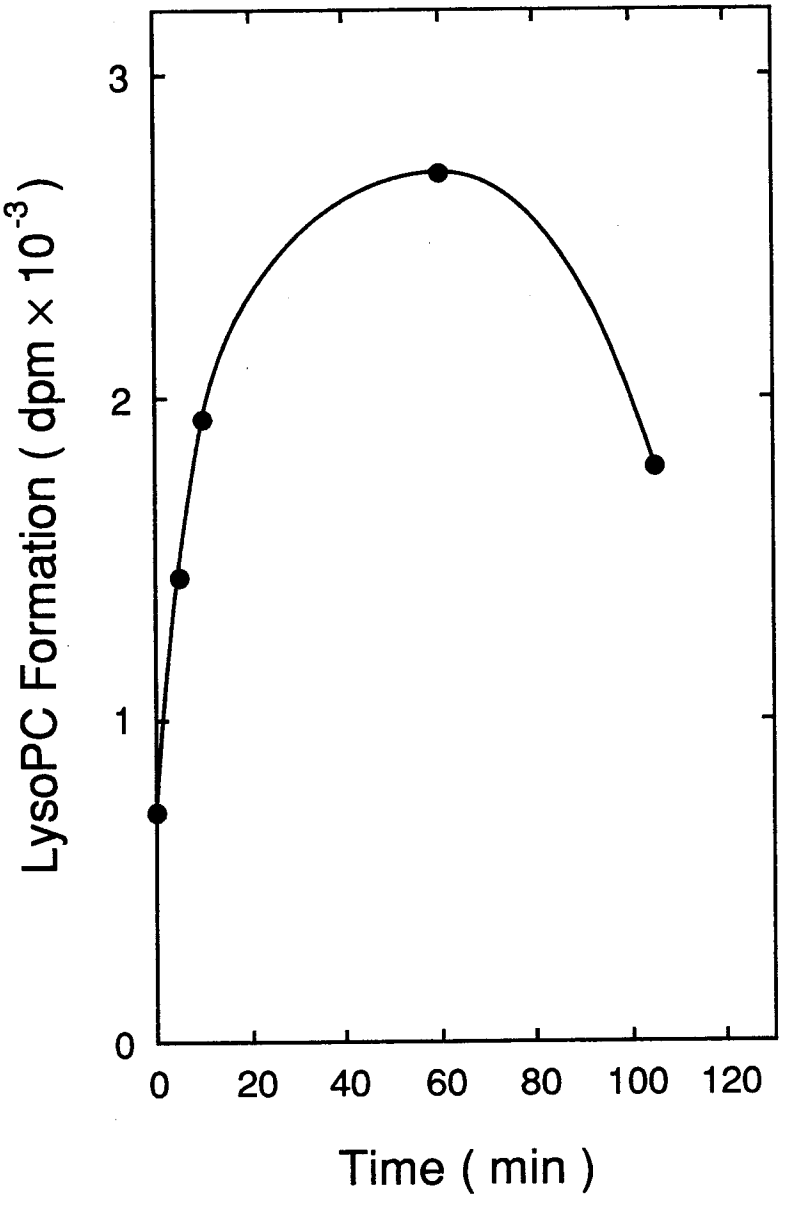

Fig. 10. Formation of lysoPC in T lymphocytes stimulated with an antigenic signal.

T lymphocytes (1×10^7 cells/ml) were labeled with [^{3}H]choline (3 μCi/ml) for 24 hr. After washing, the cells (1×10^7 cells/ml) were stimulated with an anti-CD3 monoclonal antibody (10 μg/ml). At the various times indicated, lipids were extracted and separated. The radioactivity corresponding to lysoPC was quantitated as described.

IV. Discussion

IV-1. How does lysoPC act to enhance T lymphocyte activation?

As to the role of PLA_2 in signal transduction, arachidonic acid release has been recognized so far [reviewed in (26)]. Arachidonic acid, one of *cis*-unsaturated fatty acids which is a product of phospholipids hydrolysis by PLA_2, is metabolized to produce eicosanoids such as prostaglandins which have various biological activities. However, it is reported that other *cis*-unsaturated fatty acids such as linoleic acid, like arachidonic acid, are effective for platelet activation (24). It is also noted that PLA_2, which dose not show specificity for the phospholipid acylated with arachidonic acid, is activated in response to phorbol esters in neutrophils (27). Therefore, it is attractive to suggest additional roles of PLA_2 in signal transduction. In this chapter, lysoPC, one of the products of PC hydrolysis catalyzed by PLA_2, is shown to potentiate T lymphocyte activation. However, a possibility could not be ruled out that the observed cell activation is due to some other non-specific action of lysoPC. In fact, lysoPC has a detergent-like action and shows cell-lytic activity (28) ("lyso" means lytic). In the experiments presented above, however, lysoPC does not appear to act simply as a detergent or Ca^{2+}-ionophore, since lysoPC did not induce any detectable Ca^{2+} mobilization nor did increase Ca^{2+}-sensitivity of T lymphocyte activation (Fig. 7B). Presumably, serum, which shows lipid-binding activity, traps lysoPC, and delivers this lipid slowly to the cell. It is also unlikely that lysoPC enhances penetration of 1,2-DiC_8 into the cell membranes, since lysoPC did not increase DiC_8-sensitivity of T lymphocyte activation (Fig. 7A). In addition, T lymphocytes are specifically activated by lysoPC among lysophospholipids (Fig. 6). Thus, lysoPC seems not to act simply as a non-specific membrane-perturbing reagent that modulates properties of the cell membranes.

It has been reported so far that lysoPC has several biological activities such as those to cause chemotaxis (29, 30) and relaxation of smooth muscle

(31). It is also possible that metabolites of lysoPC, rather than lysoPC itself, play some roles in cellular responses. In Fig. 8, palmitoyl-lysoPC was gradually metabolized to produce palmitic acid and PC. Palmitic acid, a saturated fatty acid, appears not to be active in signal transduction to cause cellular responses such as platelet activation (24). It has been postulated that lysoPC is acylated to PC, and subsequently hydrolyzed to produce DG by PC-specific PLC (32). However, this possibility is unlikely, since a membrane-permeant DG is essential for this cell activation, and lysoPC does not affect the sensitivity to membrane-permeant DG. Another possibility is that cellular responses induced by lysoPC may be due to its own biological agonistic action. For instance, lysophosphatidic acid (lysoPA), which is proposed to be a mitogenic signal in fibroblast, apparently acts through its own specific receptor (33), although this receptor has not been well characterized. In this study, the possibility is focused that PKC is one of the specific target for lysoPC, since the potentiation of T lymphocyte activation by lysoPC absolutely requires DG and Ca^{2+}-ionophore. Such a lysoPC action on the activity of PKC will be investigated and discussed in Chapter 2.

LysoPC accumulates in T lymphocytes slowly over 1 hr, after T lymphocyte receptor (TCR) stimulation (Fig. 10). This fact suggests that the lysoPC production from PC by PLA_2 is long-lasting, unlike DG formation from PIP_2 by PLC which is produced transiently at most for several minutes. It is possible that lysoPC acts as a sustained signal for T lymphocyte activation (Fig. 2). The metabolism of exogenous lysoPC seems to be slow (Fig. 8), presumably because this lipid is trapped by serum in the medium and delivered slowly to the cells. This slow delivery of lysoPC may mimic the sustained accumulation of this lipid when the cells are stimulated by physiological signals. The enhancement of T lymphocyte activation by lysoPC requires both membrane-permeant DG and Ca^{2+}-ionophore (Fig. 4). Delayed addition of lysoPC after DiC_8, which is very rapidly metabolized [(16), and Fig. 8], does not potentiate T lymphocyte

activation. On the other hand, the delayed addition after TPA, which is metabolically stable, always potentiates the T lymphocyte activation (Fig. 9). These results suggest that lysoPC does not enhance the pre-activated cell with DiC_8 and Ca^{2+}-ionophore, but acts in concert with DG at same time.

IV-2. Distinct actions between *cis*-unsaturated fatty acids and lysoPC.

cis-Unsaturated fatty acids, the other half of the PC molecule produced by PLA_2 action, potentiate platelet activation that is induced by DiC_8 and Ca^{2+}-ionophore (24), in a manner different from the potentiation of T lymphocyte activation by lysoPC. *cis*-Unsaturated fatty acids greatly increase the sensitivity of platelet activation to Ca^{2+}. This effect is consistent with the fact that *cis*-unsaturated fatty acids enhance the sensitivity of PKC to Ca^{2+} in a cell-free enzymatic assay (34). On the other hand, lysoPC does not increase the sensitivity of T lymphocyte activation to Ca^{2+} (Fig. 7B), although the effect of lysoPC may be mediated PKC activation. These distinct modes of the cell activation are presumably due to the difference of modes of activation of PKC enzyme by *cis*-unsaturated fatty acids and by lysoPC, as discussed in Chapter 2.

The activity of *cis*-unsaturated fatty acids and lysoPC for the potentiation of cellular responses greatly varies with the cell type examined. For the activation of platelets, lysoPC is far less effective than *cis*-unsaturated fatty acids (24), whereas for the activation of T lymphocytes, lysoPC is more effective, but *cis*-unsaturated fatty acids are ineffective as described above. In contrast, both lysoPC and *cis*-unsaturated fatty acids are effective for the potentiation of the differentiation of human promyelocytic leukemia cells (HL-60) to macrophages in the presence of DiC_8 or TPA (Asaoka, Y., Yoshida, K., and Nishizuka, Y., in preparation). The reason for such differences is not clear presently, but multiple factors may be considered. It is possible that the activity of various lipid metabolizing enzymes varies from cell to cell. For example, in platelets, the

enzymes metabolizing lysoPC might be very active, but the enzymes metabolizing *cis*-unsaturated fatty acids might be less active. In T lymphocytes, the reverse might occur. Furthermore, as the platelet activation is a short-term response, sustained signal molecules such as lysoPC may not be required. The precise molecular mechanism of the potentiation of T lymphocyte activation by lysoPC remains unclear. Further investigations are needed, and the effect of lysoPC on the PKC activation *in vitro* will be studied and discussed in Chapter 2.

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Chapter 2

Enhancement of the Activity of Protein Kinase C by Lysophosphatidylcholine

I. Introduction

I-1. PKC family in mammalian tissues and its domain structure.

PKC (ATP:protein phosphotransferase, EC 2. 7. 1. 37) was first discovered in 1977 as a proteolytically activated protein kinase present in many tissues (1). Later, it was shown to be a Ca^{2+}-activated, phospholipid-dependent enzyme (2), and was directly linked to the signal transduction pathway by the fact that 1,2-DG, one of the products of PIP_2 hydrolysis by PLC, was essential for the activation of the enzyme (3-5).

Molecular cloning and biochemical analysis has revealed that there are at least ten subspecies of PKC, and that some subspecies show distinct tissue distribution and characteristic localization within the cell [reviewed in (6, 7), Table 1]. These subspecies are categorized into three groups [(7), Fig. 11]. Group A consists of four classical or conventional PKCs (cPKC): α, βI, βII, and γ (6) . The βI and βII subspecies, which are derived from a single RNA transcript by alternative splicing, differ from each other only in ~50 amino acid residues at their carboxyl-terminal end regions, and show similar enzymological properties (8). Group B consists of four new or novel PKCs (nPKC): δ, ε, η(L), and θ (6, 9-14). Group C, which has been more recently characterized, consists of two atypical PKCs (aPKC): ζ and λ (6, 9, 15, 16) (Akimoto, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S., personal communication). The cPKC enzymes of group A have four conserved (C1 to C4) and five variable (V1 to V5) regions [(6, 7), Fig. 11] The carboxyl-terminal half, which contains C3 and C4 regions, is the protein serine/threonine kinase domain which transfers γ-phosphate of ATP to

Table 1. PKC subspecies in mammalian tissues[a].

Group	Sub-species	Molecular weight	Activators[c]	Tissue expression
A (cPKC)	α	76,799	Ca^{2+}, DG, PS, FFA	Universal
	βI	76,790	Ca^{2+}, DG, PS, FFA	Some tissues
	βII	76,933	Ca^{2+}, DG, PS, FFA	Many tissues
	γ	78,366	Ca^{2+}, DG, PS, FFA	Brain only
B (nPKC)	δ	77,517	DG, PS	Universal
	ε	83,474	DG, PS, FFA	Brain & others
	η(L)[b]	77,972	?	Lung, skin, heart
	θ[b]	81,571	?	Skeletal muscle[d]
C (aPKC)	ζ	67,740	PS, FFA	Universal
	λ[b]	67,200	?	Ovary, testis, etc.

[a]This table is adapted from review (7).

[b]The detailed enzymological properties of the η(L)-, θ-, and λ-subspecies have not yet been clarified.

[c]The activators for each subspecies are determined with calf thymus H1 histone and bovine myelin basic protein (MBP) as model phosphate acceptors.

[d]The θ-subspecies is predominantly expressed in skeletal muscle, however, expressed in other tissues such as spleen at a lower level (12).

Abbreviations: classical or conventional PKC, cPKC; new or novel PKC, nPKC; atypical PKC, aPKC; DG, 1,2-diacylglycerol; PS, phosphatidylserine; FFA *cis*-unsaturated (free) fatty acid.

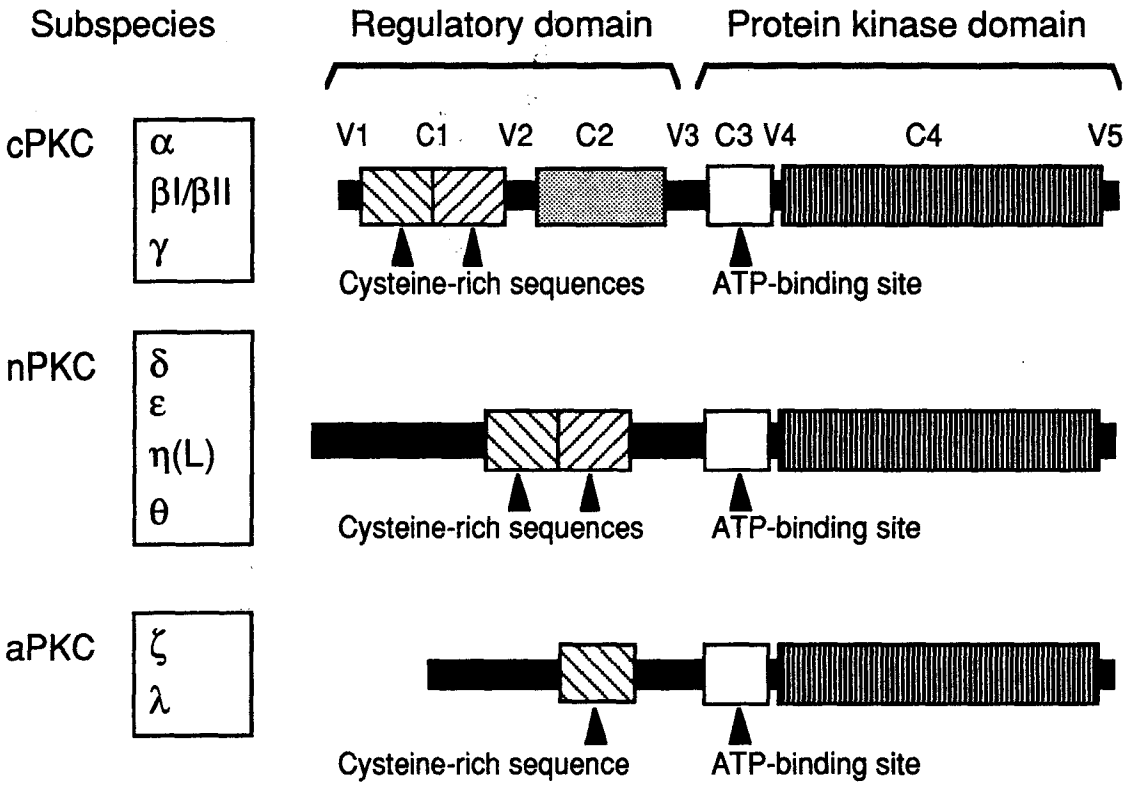

Fig. 11. Structure of PKC subspecies.

Ten subspecies of PKC and their domain structures are represented. Four conserved (C1 to C4) and five variable (V1 to V5) regions of PKC are also indicated. Details are described in the text and reviews (6, 7). The βI and βII subspecies are derived from a single gene by alternative splicing. This figure is taken from review (7).

serine or threonine residues of substrate proteins. The C3 region has an ATP-binding consensus sequence (Gly-X-Gly-X-X-Gly-·····-Lys, where X represents any amino acid) (17-19). The C4 region is probably responsible for a substrate recognition (18, 20). The amino-terminal half, which contains C1 and C2 regions, is the regulatory domain for protein kinase activity. The C1 region contains a tandem repeat of cysteine (Cys)-rich sequence, Cys-X_2-Cys-$X_{13(14)}$-Cys-X_2-Cys-X_7-Cys-X_7-Cys. Group C (aPKC) has only one set of the Cys-rich sequence (7). The polypeptide containing this Cys-rich sequence is able to bind phorbol esters, which are similar to the glycerol backbone with acyl chains of DG, with high affinity (21). Therefore, this Cys-rich sequence is probably involved in the activation by DG. This sequence resembles the consensus sequence of a "cysteine-zinc-DNA-binding finger" that is found in many metalloproteins and DNA-binding proteins that are related to transcriptional regulation (22, 23), although there is no evidence that PKC binds DNA. The C2 region, which dose not appear in nPKC and aPKC enzymes, has not been well characterized. However, it is proposed that the C2 region is responsible for Ca^{2+} activation, because the δ- and ε-subspecies of nPKC, which do not have C2 region, are Ca^{2+}-independent for their activation (10, 11), and deletion mutants of cPKC for this region obtained by mutagenesis experiments show the kinase activity as well as the phorbol-ester binding activity independent of Ca^{2+} (21, 24).

The individual patterns of expression of these ten subspecies in several tissues and cell types have been extensively investigated [reviewed in (6, 7), Table 1]. The α-, δ- and ζ-subspecies are widely distributed in many tissues and cell types. The distinct expression patterns are found for the βI- and βII-subspecies, in some cells in brain, although both subspecies are expressed in many tissues. The ε-subspecies is rich in the brain, and the γ-subspecies is expressed only in the brain. It is possible that the γ- and ε-subspecies may play crucial roles in the control of brain functions. Other subspecies have not been well investigated. However, the η(L)-subspecies has been shown to be

expressed predominantly in the lung, skin, and heart (13, 14), whereas the θ-subspecies is mainly expressed in skeletal muscle (12), and the λ-subspecies is expressed in ovary and testis (Akimoto, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S., personal communication). It is possible that these subspecies may be responsible for the control of some specific functions of these tissues and cells.

I-2. Ca^{2+} and lipid requirements of PKC subspecies.

The cPKC, nPKC, and aPKC enzymes contain several conserved regions with different combinations (Fig. 11). The cPKC is composed of C1 containing two Cys-rich sequences, C2, C3, and C4. The nPKC is composed of C1 with two Cys-rich sequences, C3, and C4, whereas C2 is absent. The aPKC has C1 with only one Cys-rich sequence, and C2 is absent. These three groups show different requirements for Ca^{2+}, DG, and phosphatidylserine (PS) as activators (Table 1). The members of cPKC are all activated by Ca^{2+}, PS, and DG or phorbol esters. The δ- and ε-subspecies of nPKC do not require Ca^{2+}, but show a PS- and DG- or phorbol ester-dependent protein kinase activity (10, 11). The ζ-subspecies of aPKC is dependent on PS but does not require Ca^{2+}, DG, nor phorbol esters for its activation (9, 15, 16). Furthermore, in the presence of PS and DG, the α-, β-, γ-, and ε-subspecies are all enhanced by *cis*-unsaturated fatty acids which are the products of PC hydrolysis by PLA_2 (11, 25). *cis*-Unsaturated fatty acids increase the sensitivity of cPKC to Ca^{2+}, in the presence of PS and DG. However, the δ-subspecies is inversely inhibited by these fatty acids (10). The γ-, ε-, and ζ- subspecies are also activated, to some extents, directly by *cis*-unsaturated fatty acids in the absence of PS and DG (11, 15, 26). Thus, it is likely that various phospholipids and their metabolites may regulate the activity of various PKC subspecies at different extents, and then induce the cellular responses. In Chapter 1, lysoPC, the other product of PC hydrolysis by PLA_2, was shown to dramatically potentiate T lymphocyte activation in the presence of a membrane permeant DG (DiC_8) and a Ca^{2+}-ionophore. Therefore, it is possible

that PKC may be one of the targets for this lysoPC action. The study in Chapter 2 describes that, in the presence of PS and DG, lysoPC has a stimulatory effect on the activity of PKC, especially cPKC, over a wide range of Ca^{2+} concentrations including physiological level.

II. Materials & Methods

II-1. Materials and chemicals.

Male Sprague-Dawley rats (7 weeks old) were employed. DE 52 (DEAE-cellulose: for anion exchange chromatography) was obtained from Whatman. AH-Sepharose 4B (aminohexyl-Sepharose, Lot. # 0107109), S-Sepharose fast flow (for cation exchange chromatography), Mono Q HR 5/5 (for anion exchange chromatography) were purchased from Pharmacia-LKB. TSK gel phenyl-5PW (for hydrophobic chromatography) and heparin-5PW were purchased from Tosoh (Tokyo). Hydroxyapatite column (KB-0515 type C) was purchased from Koken, Ltd. (Tokyo). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF) was obtained from Wako Pure Chemical (Osaka). Leupeptin was a product of Peptide Institute (Osaka). [γ-^{32}P]ATP was a product of New England Nuclear. Calf thymus H1 histone was prepared as described (27). Bovine myelin basic protein (MBP), L-α-caproyllysoPC, L-α-myristoyllysoPC, L-α-palmitoyllysoPC, L-α-stearoyllysoPC, L-α-palmitoyllysoPE, L-α-lysoPS (from bovine brain) and L-α-lysoPI (from soy bean) were obtained from Sigma. PS (from bovine brain), 1,2-diolein, and α-palmitoyllysoPA were purchased from Serdary Research Laboratories (London, ON, Canada). PS and diolein were dissolved in chloroform, and lysophospholipids were dissolved in a mixture of chloroform and methanol (1:1), respectively. These lipids were stored in dark at -20°C until use. Other chemicals were purchased from commercial sources.

II-2. Purification of α-, β-, and γ-subspecies of PKC from rat brain.

II-2-1. Buffers.

Homogenization Buffer.

20	mM	Tris-HCl (pH 7.5)
10	mM	EGTA
2	mM	EDTA
250	mM	sucrose

The protease inhibitors listed below are added immediately before use.

50	μM	*p*-APMSF
20	μg/ml	leupeptin

Buffer A.

20	mM	Tris-HCl (pH 7.5)
0.5	mM	EGTA
0.5	mM	EDTA
10	mM	2-mercaptoethanol

2-mercaptoethanol is added immediately before use.

Buffer B.

20	mM	potassium phosphate (pH 7.5)
0.5	mM	EGTA
0.5	mM	EDTA
10	% (vol/vol)	glycerol
10	mM	2-mercaptoethanol

2-mercaptoethanol is added immediately before use.

Stock Buffer.

20	mM	Tris-HCl (pH 7.5)
0.5	mM	EGTA
0.5	mM	EDTA
10	% (vol/vol)	glycerol
0.02	% (vol/vol)	Triton X-100
10	mM	2-mercaptoethanol

2-mercaptoethanol is added immediately before use.

II-2-2. Procedure.

Step 1. The α-, β-, and γ-subspecies were purified from rat brain as described (26). The procedures were schematically summarized in Fig. 13. Fifty-two rats were decapitated and the brains were quickly removed with scissors. The following manipulations were carried out at 0–4°C. After the brains were rinsed in homogenization buffer, every 4–5 brains (≈ 5 g, wet

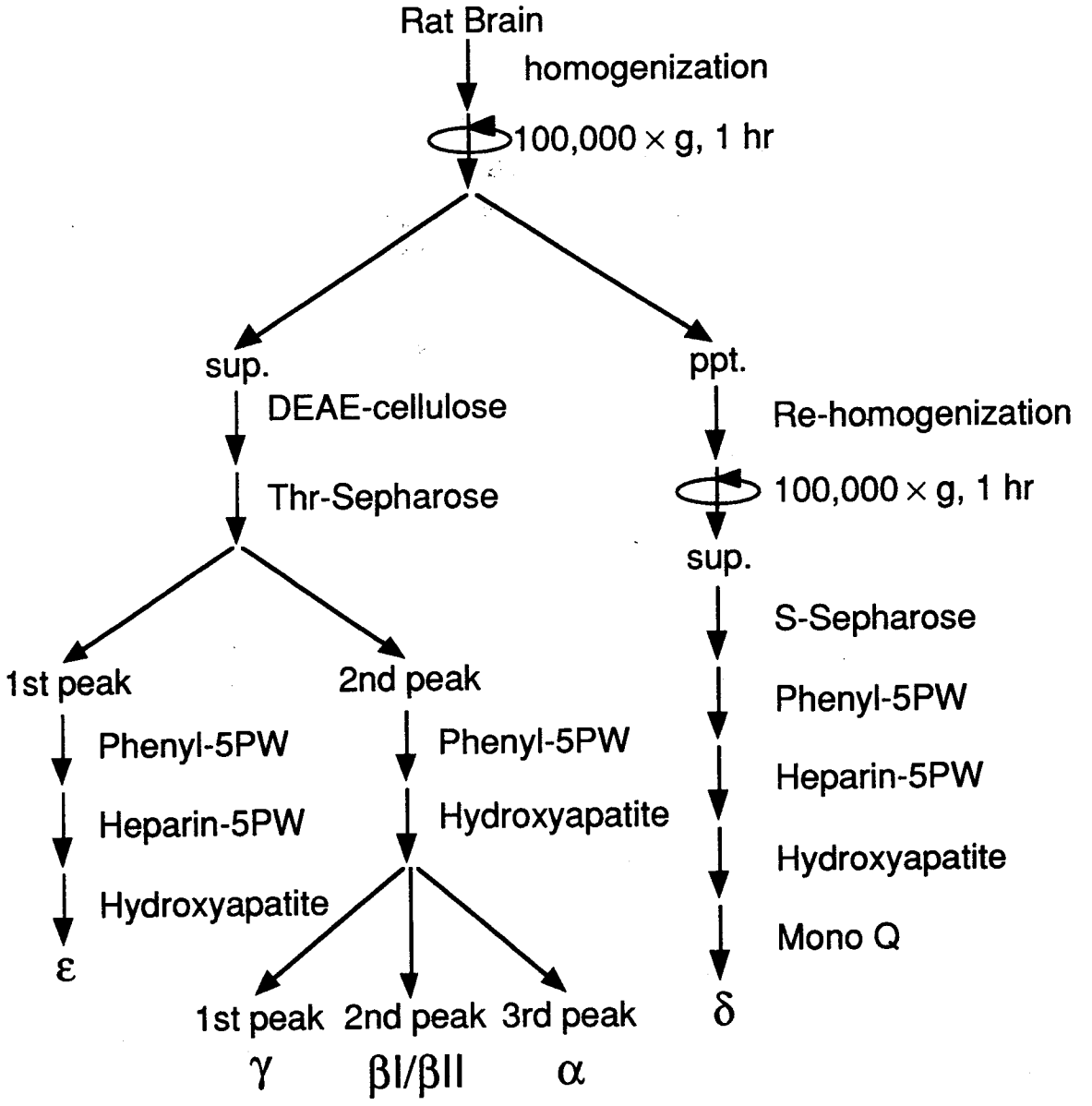

Fig. 12. Schematic representation of the purification procedures for PKC subspecies.

The purification procedures for the α- to ε-subspecies are represented. All procedures were performed at 0–4°C. sup., supernatant; ppt, precipitate; Thr-Sepharose, Threonine-Sepharose. Details are described in the text.

weight) were homogenized in a Potter-Elvehjem Teflon/glass homogenizer (5–6 strokes) with 20 ml of the homogenization buffer. The homogenates were collected (320 ml) and centrifuged for 60 min at 100,000 × g in 45 Ti rotor (Beckman). The supernatant was used for purification of the α-, β-, γ-, and ε-subspecies, and the precipitate was used for purification of the δ-subspecies (to II-3).

Step 2. The supernatant (265 ml) was applied to a DEAE-cellulose (DE 52) column (5.2 × 11.8 cm: 250 ml) equilibrated with buffer A. The column was washed with 500 ml of the same buffer, and then with 2.5 liters of buffer A containing 20 mM NaCl. The α-, β-, γ-, and ε-subspecies were co-eluted batchwise from the column with 150 mM NaCl. The first 125 ml (1/2 column volume) was discarded, and next 625 ml (2.5 column volume) was collected.

Step 3. The DEAE-cellulose fraction (625 ml) was diluted with 2.5 liters (4 volumes) of buffer A and applied to a threonine-Sepharose column (1.6 × 11 cm: 22 ml) equilibrated with buffer A. Threonine-Sepharose was prepared to couple L-threonine to AH-Sepharose as described (28). An FPLC system (Pharmacia-LKB) was employed for all subsequent column chromatographies. The column was washed with 40 ml of buffer A at a flow rate of 1 ml/min and eluted with a 320-ml linear concentration gradient of NaCl (0–0.4 M) in buffer A at a flow rate of 2 ml/min, followed by elution with an 80-ml linear concentration gradient of NaCl (0.4–1 M) in buffer A, and then with 200 ml of 1 M NaCl in buffer A. Fractions (10 ml each) were collected and assayed for PKC activity by micro assay method as described in II-5-1 (in following procedures, PKC assays were performed by micro assay method). The PKC activity was detected as two peaks. The first peak (fractions 14–25) showed DG-dependent, Ca^{2+}-independent PKC activity, and contained the ε-subspecies. The second peak (fractions 35–60) showed DG and Ca^{2+}-dependent PKC activity, and contained the α-, β-, and γ-subspecies. The first peak was used for purification of the ε-

subspecies (to II-4-2), and the second peak was used for purification of the α-, β-, and γ-subspecies as described below.

Step 4. The threonine-Sepharose second peak fractions (35–60: 360 ml) were applied directly to a TSK gel phenyl-5PW column (2.15 × 15 cm: 54.5 ml) equilibrated with buffer A containing 1 M NaCl. The column was washed with 80 ml of buffer A containing 1 M NaCl, and then eluted with a 320-ml decreasing linear concentration gradient of NaCl (1–0 M) in buffer A at a flow rate of 4 ml/min. Fractions (8 ml each) were collected, and assayed for PKC activity. The PKC activity was eluted in fractions 30–39.

Step 5. The phenyl-5PW fractions (80 ml) were applied directly to a hydroxyapatite column (0.78 × 15 cm: 7.2 ml) equilibrated with buffer B. The column was washed with 30 ml of buffer B, and then eluted with an 84-ml linear concentration gradient of potassium phosphate (20–215 mM) in buffer B at a flow rate of 0.4 ml/min. Fractions (1 ml each) were collected and assayed. Three peaks of PKC activity were detected in fractions 27–32 (peak I, the γ-subspecies), 35–41 (peak II, the β-subspecies), and 53–64 (peak III, the α-subspecies). Since the βI- and βII-subspecies have not been separated chromatographically, the β-subspecies, in this study, means the mixture of the βI- and βII-subspecies. These peaks were dialyzed against the stock buffer for 3 hr, and then stored at –80°C.

II-3. Purification of δ-subspecies of PKC from rat brain.

II-3-1. Buffers.

Suspension Buffer.

20	mM	Mes-NaOH (pH 6.5)
10	mM	EGTA
2	mM	EDTA
250	mM	sucrose
1	% (vol/vol)	Triton X-100

The protease inhibitors listed below are added immediately before use.

50	μM	*p*-APMSF
20	μg/ml	leupeptin

Buffer S.

20	mM	Mes-NaOH (pH 6.5)
0.5	mM	EGTA
0.5	mM	EDTA

These reagents listed below are added immediately before use.

10	mM	2-mercaptoethanol
50	μM	*p*-APMSF
20	μg/ml	leupeptin

Buffer A.

For the purification of δ-subspecies, the protease inhibitors listed below are added immediately before use, to buffer A described in II-2-1.

50	μM	*p*-APMSF
20	μg/ml	leupeptin

Buffer B.

For the purification of δ-subspecies, these reagents listed below are added immediately before use, to buffer B described in II-2-1.

50	μM	*p*-APMSF
20	μg/ml	leupeptin
0.02	% (vol/vol)	Triton X-100

Buffer C.

20	mM	Tris-HCl (pH 7.5)
0.5	mM	EGTA
0.5	mM	EDTA
60	% (vol/vol)	glycerol

these reagents listed below are added immediately before use.

0.12	% (vol/vol)	Triton X-100
10	mM	2-mercaptoethanol
50	μM	*p*-APMSF
20	μg/ml	leupeptin

Stock Buffer.

For the stock of δ-subspecies, glycerol is used at the concentration of 20% (vol/vol), and the protease inhibitors listed below are added immediately before use, to stock buffer described in II-2-1.

50	μM	*p*-APMSF
20	μg/ml	leupeptin

II-3-2. Procedure.

Step 1. The δ-subspecies was purified from rat brain as described (10). Step 1, including homogenization and centrifugation, was the procedure described in II-2-2 step 1.

Step 2 . The precipitate from II-2-2 step 1 was used as starting material in this procedure. The precipitate is able to be stored directly at –80°C during the purification of other subspecies. The precipitate in each centrifuge tube was re-homogenized in 25 ml of suspension buffer with a Potter-Elvehjem Teflon/glass homogenizer (5–6 strokes). The homogenates were collected and gently stirred for 1 hr. The homogenate (230 ml) was centrifuged for 60 min at 100,000 × g in 45 Ti rotor (Beckman). The supernatant (160 ml) was applied to an S-Sepharose fast flow column (3 × 11 cm: 77.7 ml) equilibrated with buffer S. The column was washed with 777 ml (10 column volume) of buffer S containing 20 mM NaCl, and then eluted batchwise with 240 ml (≈ 3 column volume) of buffer S containing 400 mM NaCl. The first 20 ml (≈ 1/4 column volume) was discarded, and the next 220 ml (≈ 2.75 column volume) was collected. The PKC activity dependent on PS and diolein could not be detected in this eluate, however, the δ-subspecies was detected by immunoblot analysis with the antibody against a δ-subspecies synthetic peptide as describes below.

Step 3. The S-Sepharose eluate (220 ml) was adjusted to 1 M NaCl and pH 7.5, by the addition of 7.78 g of solid NaCl and 1M Tris-HCl (pH 9.5). For further purification, all columns were equipped with an FPLC system (Pharmacia-LKB). This enzyme solution was applied to a TSK gel phenyl-5PW column (2.15 × 15 cm: 54.5 ml) equilibrated with buffer A containing 1 M NaCl.

The column was washed with 80 ml of buffer A containing 1 M NaCl, and then eluted with a 360-ml decreasing linear concentration gradient of NaCl (1–0 M) in buffer A at flow rate of 4 ml/min. Fractions (8 ml each) were collected and assayed for PKC activity. A broad peak appeared, which was not absolutely dependent on PS and diolein. However, immunoblot analysis of each fraction showed a peak that was immunoreactive with the antibody against a δ-subspecies synthetic peptide. The fractions of this immunoreactive peak (fractions 26–36), which also contained several other PKC subspecies, were pooled.

Step 4. The Phenyl-5PW peak fractions (88 ml) were diluted with 400 ml (4.5 volumes of the fractions) of buffer A and applied to a TSK gel heparin-5PW column (2.15 × 15 cm: 54.5 ml) equilibrated with buffer A. The column was washed with 80 ml of buffer A, and then eluted with a 360-ml linear concentration gradient of NaCl (0–0.35 M) in buffer A at a flow rate of 4 ml/min. Each Fraction (6 ml) was collected together with 1 ml of buffer C to stabilize the enzymatic activity. The PKC activities were separately eluted in fractions 17–23 and 41–59. The first peak (fractions 17–23) was reactive with the antibody against a δ-subspecies synthetic peptide and showed Ca^{2+}-independent PKC activity. The second peak (fractions 41–59) was not reactive with the δ-specific antibody and showed Ca^{2+}-dependent PKC activity. The identification of the second peak remains to be clarified. The δ-subspecies was not separated from other subspecies until this step of purification.

Step 5. The first peak of heparin-5PW (fractions 17–23: 42 ml) was applied directly to a hydroxyapatite column (0.78 × 15 cm: 7.2 ml) equilibrated with buffer B. The column was washed with 30 ml of buffer B, and then eluted with an 84-ml linear concentration gradient of potassium phosphate (20–215 mM) in buffer B at a flow rate of 0.4 ml/min. Fractions (1 ml each) were collected and assayed. The PKC activity was eluted in fractions 39–46.

Step 6. The hydroxyapatite fractions (8 ml) were pooled and dialyzed against buffer B for 3 hr, 2 times. The dialyzate was applied onto a Mono Q HR5/5 column (0.5 × 5 cm: 1 ml) equilibrated with buffer B. After washing with 20 ml of buffer B, the column was eluted by application of a 50-ml linear concentration gradient of NaCl (0–0.35 M) in buffer B. Fractions (1 ml each) were collected and assayed. The PKC activity was eluted in fractions 19–26. These fractions (7 ml) were combined and dialyzed against stock buffer for 3 hr. The dialyzate was stored at –80°C.

II-4. Purification of ε-subspecies of PKC from rat brain.

II-4-1. Buffers.

Buffer A, Buffer B, and Stock buffer.

For purification of the ε-subspecies, the protease inhibitors listed below are added immediately before use, to buffer A, B, and stock buffer described in II-2-1.

5	μM	*p*-APMSF
2	μg/ml	leupeptin

II-4-2. Procedure.

Steps 1–3. The ε-subspecies was purified from rat brain as described (11). Steps 1–3 were the procedures described in II-2 steps 1–3, including homogenization, centrifugation, DEAE-cellulose column chromatography, and threonine-Sepharose column chromatography.

Step 4. The threonine-Sepharose first peak fractions (14–25: 120 ml) from II-2-2 step 3 were used as starting material in this procedure. These fractions were collected and adjusted to NaCl concentration of 0.83 M by the addition of 24 ml of 4 M NaCl and 2.7 ml of 10 × buffer A, and then applied to a TSK gel phenyl-5PW column (2.15 × 15 cm: 54.5 ml) equilibrated with buffer A containing 1 M NaCl. The column was washed with 80 ml of buffer A containing 1 M NaCl, and then eluted with a 320-ml decreasing linear concentration gradient of NaCl (1–0 M) in buffer A at a flow rate of 4 ml/min. Fractions (8 ml

each) were collected and assayed for PKC activity. The PKC activity was eluted in fractions 27–32.

Step 5. The phenyl-5PW fractions (48 ml) were diluted with 336 ml (7 volumes of the fractions) of buffer A and applied to a TSK gel heparin-5PW column (2.15 × 15 cm: 54.5 ml) equilibrated with buffer A. The column was washed with 80 ml of buffer A, and then eluted with a 480-ml linear concentration gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 3 ml/min. Fractions (6 ml each) were collected and assayed. The PKC activity was eluted in fractions 26–34.

Step 6. The heparin-5PW fractions (54 ml) were applied directly to a hydroxyapatite column (0.78 × 15 cm: 7.2 ml) equilibrated with buffer B. The column was washed with 30 ml of buffer B, and then eluted with an 84-ml linear concentration gradient of potassium phosphate (20–215 mM) in buffer B at a flow rate of 0.4 ml/min. Fractions (1 ml each) were collected and assayed. The PKC activity was eluted in fractions 35–41. Fractions 36–39 (4 ml) were combined, and dialyzed against stock buffer for 3 hr. The dialyzate was stored at –80°C.

II-5. PKC assay.

II-5-1. Micro assay method.

PKC activity was assayed for detection of the peak fractions in purification procedures, as described (10) with slight modifications. Briefly, the standard reaction mixture (50 µl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ (for cPKC) or 0.5 mM EGTA (for nPKC), 10 µM [γ-^{32}P]ATP (2–10 × 10^3 cpm/pmol), 10 µg of calf thymus H1 histone (for cPKC) or myelin basic protein (MBP, for nPKC) as phosphate acceptors, the enzyme, and mixed liposomes composed of PS (8 µg/ml) and 1,2-diolein (0.8 µg/ml). The mixed liposomes were prepared as follows. PS and diolein were first mixed together in an organic solvent, and dried under a nitrogen stream. The residue was then sonicated in 20 mM Tris-HCl (pH 7.5). The reaction was started by the addition of

approximate volume of a mixture containing Tris-HCl (pH 7.5), $MgCl_2$, [γ-^{32}P]ATP, and a phosphate acceptor, in an Eppendorf tube containing the enzyme, mixed liposomes, and calcium. Basal activity was measured in the presence of 0.5 mM EGTA, without PS and diolein. After incubation for 3–10 min at 30°C, the reaction was terminated by spotting 40-µl of the reaction mixture onto 2 cm × 2 cm P81 paper (Whatman). The papers were washed five times, for 5 min each, by immersion in 75 mM H_3PO_4 in bat (1 ml/P81 paper), with shaking. The P81 paper was transferred into a vial containing 10 ml of water, and then the radioactivity was quantitated by Cerenkov counting. One unit of cPKC, the α-, β-, and γ-subspecies, was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{32}P into H1 histone per min at 30°C (29). One unit of nPKC, the δ- and ε-subspecies, was defined as above, except for MBP instead of H1 histone (10, 11).

II-5-2. PKC assay for lysophospholipid.

The enzyme activity was assayed as described (29) with some modifications. Briefly, the standard reaction mixture (0.25 ml) contained 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 1 µM $CaCl_2$, 10 µM [γ-^{32}P]ATP (150–250 cpm/pmol for cPKC, 500–700 cpm/pmol for nPKC), 50 µg of calf thymus H1 histone, the enzyme, and mixed liposomes composed of PS (1 µg/ml), 1,2-diolein (2.5 µg/ml), and L-α-palmitoyl-lysoPC (4 µM) unless otherwise indicated in each experiment. The mixed liposomes were prepared as follows: PS, diolein, and lysophospholipid were mixed first together in the organic solvents, and dried under a nitrogen stream. The residue was then sonicated in 20 mM Tris-HCl (pH 7.5) and added directly to the reaction mixture. The reaction was started by the addition of the enzyme (in the case of cPKC, ≈ 0.05 unit; in the case of nPKC, ≈ 0.002 unit). After incubation for 5 min (for cPKC) or 10 min (for nPKC) at 30°C, the reaction was stopped by the addition of 25% (w/v) trichloroacetic acid, and the sample was poured onto a nitrocellulose filter (pore

size: 0.45 μm, Advantec, Tokyo) which was set in a vacuum apparatus. The nitrocellulose filter was washed with 25% (w/v) trichloroacetic acid, 3 times, and then the acid-precipitable materials were trapped. The nitrocellulose filter was transferred into a vial including 10 ml of water, and then the radioactivity was quantitated by Cerenkov counting.

II-5-3. Phosphorylation of MARCKS protein by PKC.

The phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein by PKC was performed as described in micro assay method (II-5-1) with some modifications. Briefly, the standard reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 μM $CaCl_2$, 10 μM [γ-^{32}P]ATP (1×10^4 cpm/pmol), partially purified bovine MARCKS protein as described in II-6, the α-subspecies of PKC (about 0.05 unit), and mixed liposomes composed of PS (1 μg/ml), 1,2-diolein (2.5 μg/ml), and L-α-palmitoyllysoPC (4 μM). The mixed liposomes were prepared as described under II-5-2. The reaction was started by the addition of the enzyme. After incubation for 15 min at 30°C, the reaction was stopped by the addition of the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS/PAGE, (30)]. The phosphorylated proteins were separated with SDS/PAGE using 5–20% gradient gel. The radioactivity of the protein band corresponding to MARCKS was quantitated with a BAS-2000 Bioimage analyzer (Fuji Film, Tokyo).

II-6. Partial purification of bovine MARCKS protein.

II-6-1. Buffers.

Homogenization buffer.

10	mM	Tris-HCl (pH 7.5)
250	mM	sucrose
10	mM	dithiothreitol
2	mM	EGTA

The protease inhibitors listed below are added immediately before use.

50	μM	*p*-APMSF
10	μg/ml	leupeptin

Suspension buffer.

50	mM	Tris-HCl (pH 7.5)
150	mM	NaCl

The protease inhibitors listed below are added immediately before use.

50	μM	*p*-APMSF
10	μg/ml	leupeptin

II-6-2. Procedure.

Bovine MARCKS protein was purified as described (31) with modifications. Bovine brain was obtained from a slaughterhouse. The bovine brain (600 g) stored at –80°C was thawed, and homogenized with four 30 sec bursts with a polytron homogenizer in 3,000 ml of homogenization buffer. The homogenate was centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was boiled for 10 min, cooled to 4°C, and centrifuged at 12,000 × g for 30 min at 4°C. This supernatant was collected, and 100% (w/v) trichloroacetic acid was added to a final concentration of 1% (w/v). The mixture was stirred for 1 hr at 4°C, and then centrifuged at 12,000 × g for 20 min at 4°C. The resulting supernatant was acid-precipitated by the addition of 100% (w/v) trichloroacetic acid to a final concentration of 15% (w/v), and the mixture was stirred for 3 hr at 4°C. Following centrifugation at 12,000 × g for 30 min at 4°C, the precipitate was suspended in suspension buffer, and adjusted to pH 7.5 by the addition of 5 M NaOH. This suspension was stored at –20°C.

II-7. Antibodies and immunoblot analysis.

II-7-1. Antibodies against PKC subspecies.

Five polyclonal antibodies were employed, which were raised against synthetic peptides that are parts of the deduced amino acid sequences of the rat brain PKC, as described (10, 11, 32). Antibody CKp$V_5\alpha$-a was raised against a peptide corresponding to a part of V5 region of the α-subspecies (sequence 662–672, Gln-Phe-Val-His-Pro-Ile-Leu-Gln-Ser-Ala-Val); CKp$V_1\beta$-a was raised against a peptide corresponding to a part of V1 region of the β-subspecies (sequence 4–19, Pro-Ala-Ala-Gly-Pro-Pro-Pro-Ser-Glu-Gly-Glu-Glu-Ser-Thr-Val-Arg); CKp$V_5\gamma$-a was raised against a peptide corresponding to a part of V5 region of the γ-subspecies (sequence 684–696, Asp-Ala-Arg-Ser-Pro-Thr-Ser-Pro-Val-Pro-Val-Pro-Val); CKp$V_3\delta$-a was raised against a peptide corresponding to a part of V3 region of the δ-subspecies (sequence 301–317, Lys-Pro-Glu-Thr-Pro-Glu-Thr-Val-Gly-Ile-Tyr-Gln-Gly-Phe-Glu-Lys-Lys); CKp$V_5\varepsilon$-a was raised against a peptide corresponding to a part of V5 region of the ε-subspecies (sequence 714–735, Glu-Ala-Ile-Val-Lys-Gln-Ile-Asn-Gln-Glu-Glu-Phe-Lys-Gly-Phe-Ser-Tyr-Phe-Gly-Glu-Asp-Leu).

II-7-2. Immunoblot analysis.

Immunoblot analysis was carried out as described (33) with some modifications. Briefly, after electrophoresis on an 8% polyacrylamide gel, the proteins and prestained molecular weight markers (BRL) were electrotransferred to a nylon membrane (Immobilon, Millipore). The membrane was blocked with 3% gelatin, and incubated with each anti-PKC subspecies antibody solution as a first antibody. This membrane was treated with a horseradish peroxidase-conjugated second antibody (Bio-Rad). Detection was performed with diaminobenzidine. The prestained molecular weight markers were composed of myosin heavy chain (200 k), phosphorylase b (97.4 k), bovine serum albumin (68.0 k), ovalbumin (43.0 k), carbonic anhydrase (29.0 k), β-lactoglobulin (18.4 k), and lysozyme (14.3 k).

II-8. Other procedures.

SDS/PAGE was performed as described (30). Molecular weight markers (Sigma) for Coomassie brilliant blue (CBB) staining were composed of myosin heavy chain (205 k), β-galactosidase (116 k), phosphorylase b (97.4 k), albumin (bovine, 66.0 k), albumin (egg, 45.0 k), and carbonic anhydrase (29.0 k). Proteins were determined by the method of Bradford (34) with bovine serum albumin as a standard.

III. Results

III-1. Purity of PKC subspecies used in this study.

The α-, β-, γ-, δ-, and ε-subspecies (cPKC, 300 ng each; nPKC, 140 ng each) purified as described in Materials & Methods were analyzed with SDS/PAGE (Fig. 13A). All subspecies were purified to near homogeneity, as judged by CBB staining. The purity of each subspecies was confirmed also by silver staining (data not shown). The δ-subspecies was detected as doublet bands, as previously reported (10). These doublet proteins appeared to be due to different phosphorylation states and their enzymatic activities were not distinguishable from each other (10). The ε-subspecies was also showed as doublet bands, as judged by silver staining (data not shown), as previously reported (11). Further, each subspecies (140 ng) was subjected to the immunoblot analysis (Fig. 13B–F). All subspecies were recognized specifically by the respective specific antibodies. Therefore, all PKC preparations employed in the subsequent studies were shown to be homogeneous.

III-2. Effect of lysoPC on cPKC activity.

The effect of lysoPC on each member of the PKC family was studied by measuring its activity with H1 histone as a phosphate acceptor protein in the presence of PS and 1,2-diolein as DG. Without lysoPC, cPKC showed maximum activity at 1×10^{-4}-M $CaCl_2$, in the presence of PS and DG (Fig. 14A). Even at physiologically low concentrations of $CaCl_2$, below 1×10^{-6} M, cPKC showed the enzymatic activity dependent on PS and DG, as previously shown (26). LysoPC at 4 μM enhanced the activity of the α-, β-, and γ-subspecies of cPKC over a wide range of Ca^{2+} (Fig. 14A). Higher concentrations of lysoPC, such as 16 μM, enhanced the activity of cPKC more effectively in the presence of high concentrations of $CaCl_2$, whereas inhibited the activity when the concentrations of this divalent cation were below 1×10^{-5} M (Fig. 14A). The effect of various

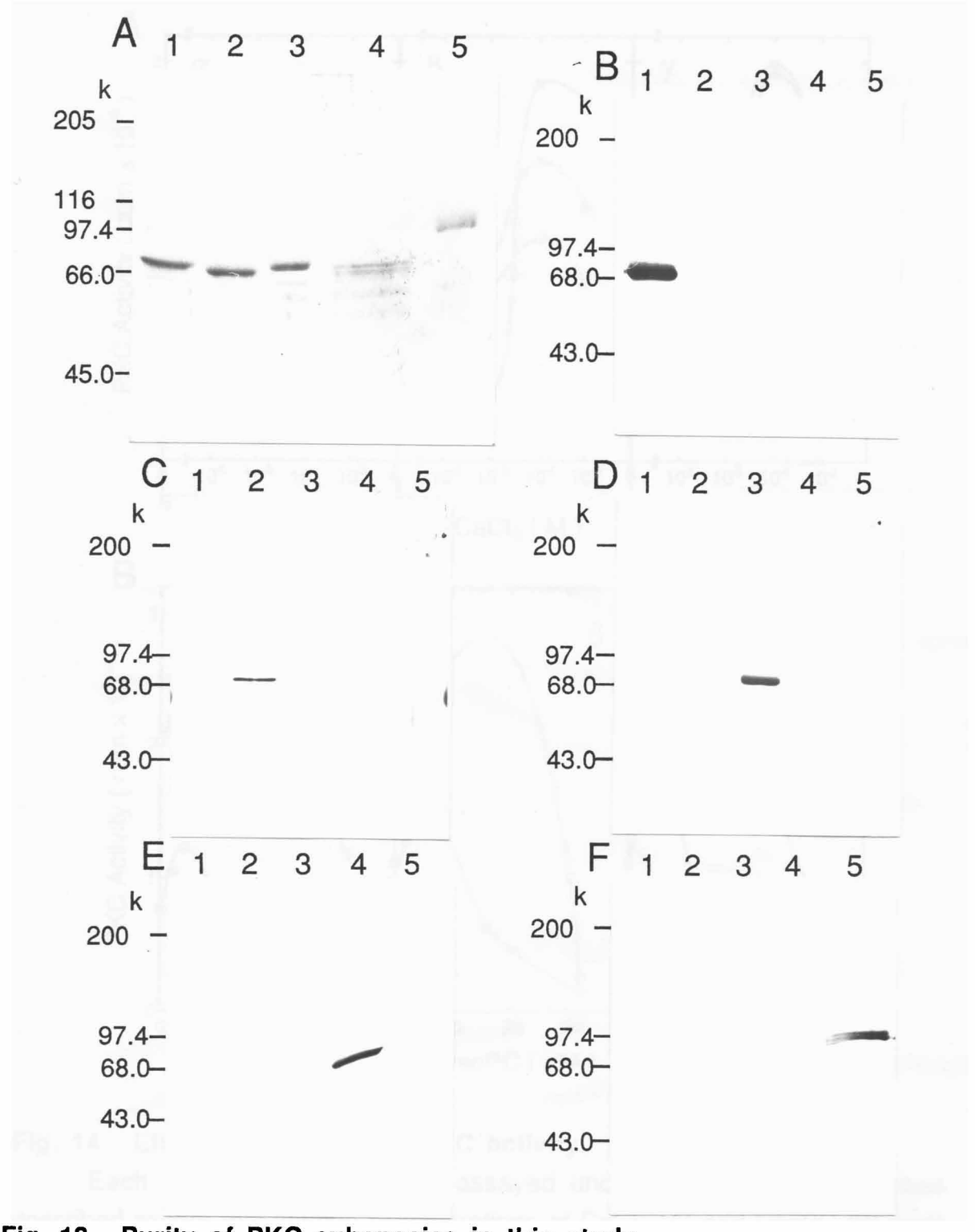

Fig. 13. Purity of PKC subspecies in this study.

The subspecies purified as described in Materials & Methods were analyzed by SDS/PAGE using 8% gel, followed by immunoblotting. 1, the α-subspecies; 2, the β-subspecies; 3, the γ-subspecies; 4, the δ-subspecies; 5, the ε-subspecies. A, CBB staining. B–F, Immunoblot analysis with antibody CKpV$_5$α-a (recognizes the α-subspecies), CKpV$_1$β-a (the β-subspecies), CKpV$_5$γ-a (the γ-subspecies), CKpV$_3$δ-a (the δ-subspecies), and CKpV$_5$ε-a (the ε-subspecies). Details are described in Materials & Methods and Results sections.

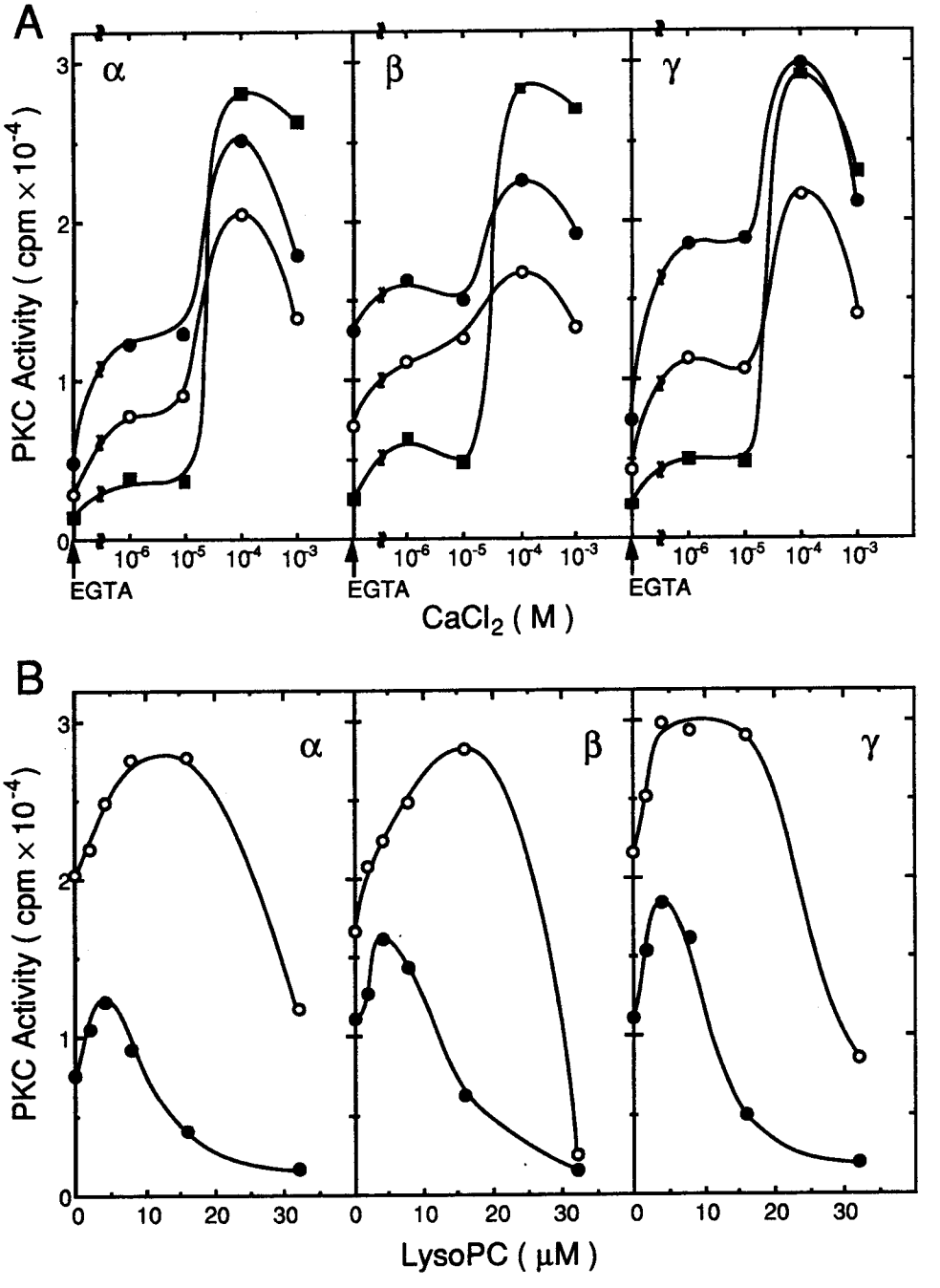

Fig. 14. Effect of lysoPC on cPKC activity.

Each cPKC subspecies was assayed under the standard conditions described except that various concentrations of $CaCl_2$ (A) and lysoPC (B) were added as indicated. α, the α-subspecies; β, the β-subspecies; γ, the γ-subspecies. A, Effect of various concentrations of $CaCl_2$ on the activation of cPKC by lysoPC. EGTA (5 mM) instead of $CaCl_2$ was added to the reaction mixtures where indicated by arrows. ●, 4-μM lysoPC; ■, 16-μM lysoPC; ○, no addition. B, Effect of various concentrations of lysoPC on cPKC activity. ●, 1×10^{-6} M $CaCl_2$; ○, 1×10^{-4} M $CaCl_2$.

concentrations of lysoPC on the cPKC activity was investigated further. In the presence of 1×10^{-6} M $CaCl_2$, lysoPC at 4 μM enhanced the activity of the α-subspecies most effectively, but in the presence of 1×10^{-4} M $CaCl_2$, the maximum enzymatic activity was obtained at 16-μM lysoPC (Fig. 14B). At both $CaCl_2$ concentrations, higher concentrations of lysoPC markedly inhibited the enzymatic activity (Fig. 14B). Similar results were obtained for the β- and γ-subspecies (Fig. 14B). These results indicated that lysoPC biphasically regulates the cPKC activity, stimulatory and inhibitory. Namely, lysoPC at lower concentrations, such as 4 μM, has stimulatory effect on cPKC activity over a wide range of Ca^{2+} concentrations including at physiological concentrations. In contrast, at higher concentrations, lysoPC has inhibitory effect especially at low concentrations of Ca^{2+}.

III-3. Effect of lysoPC on nPKC activity.

The activity of the δ-subspecies was enhanced slightly by lysoPC at 4 μM and inhibited at 16 μM, irrespective of Ca^{2+} concentrations. On the other hand, the activity of the ε-subspecies was inhibited in proportion to the concentration of lysoPC (Fig. 15A). Both the δ- and ε-subspecies, which are independent on Ca^{2+} (10, 11), did not show any Ca^{2+}-dependent enzymatic activity irrespective of the concentrations of lysoPC. Fig. 15B also shows that lysoPC has an inhibitory rather than stimulatory effect on the activity of the δ- and ε-subspecies.

III-4. DG and PS concentrations.

To investigate the mode of enhancement of cPKC activity by lysoPC, the effects of DG and PS on PKC activity were examined. In the presence and absence of lysoPC, the activation of cPKC by various concentrations of DG was measured with PS (1 μg/ml) and $CaCl_2$ (1×10^{-6} M). In the presence of 4-μM lysoPC, the activity of the α-subspecies was enhanced maximally by diolein at

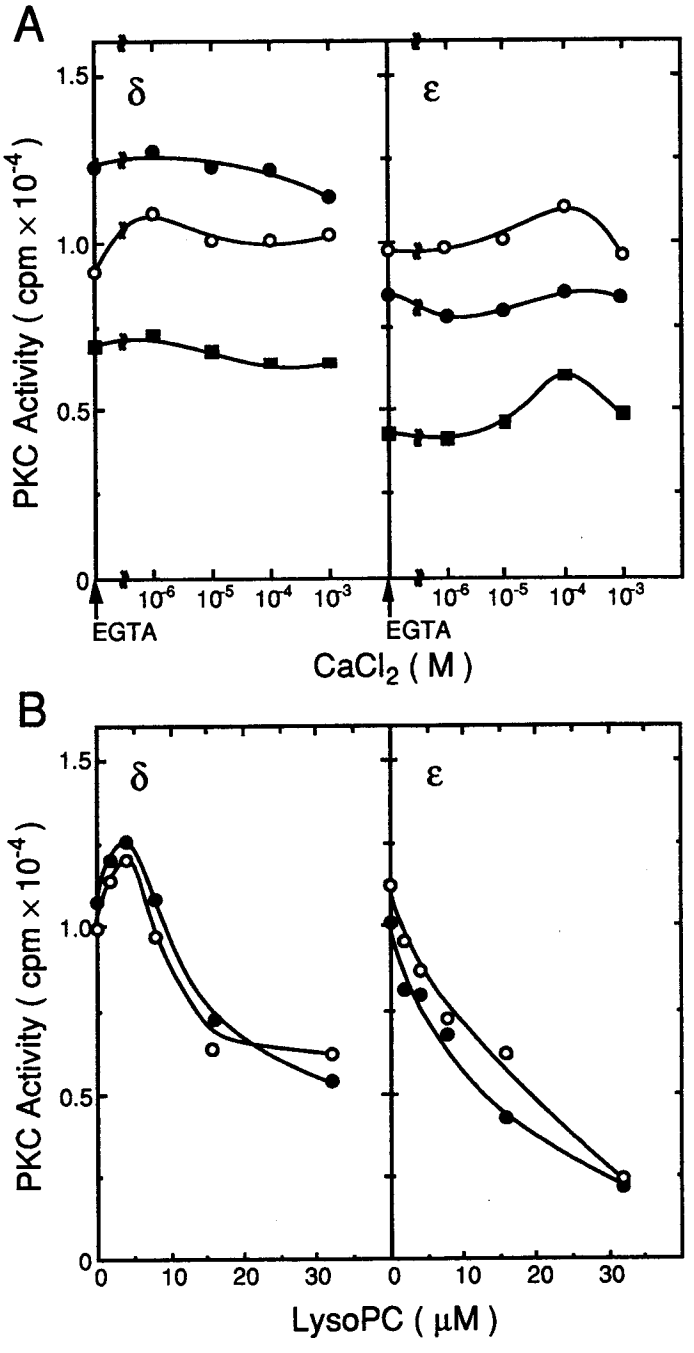

Fig. 15. Effect of lysoPC on nPKC activity.

Each nPKC subspecies was assayed under the standard conditions except that various concentrations of $CaCl_2$ (A) and lysoPC (B) were added as indicated. δ, the δ-subspecies; ε, the ε-subspecies. A, Effect of various concentrations of $CaCl_2$ on the activation of nPKC by lysoPC. EGTA (5 mM) instead of $CaCl_2$ was added to the reaction mixtures where indicated by arrows. ●, 4-μM lysoPC; ■, 16-μM lysoPC; ○, no addition. B, Effect of various concentrations of lysoPC on nPKC activity. ●, 1×10^{-6} M $CaCl_2$; ○, 1×10^{-4} M $CaCl_2$.

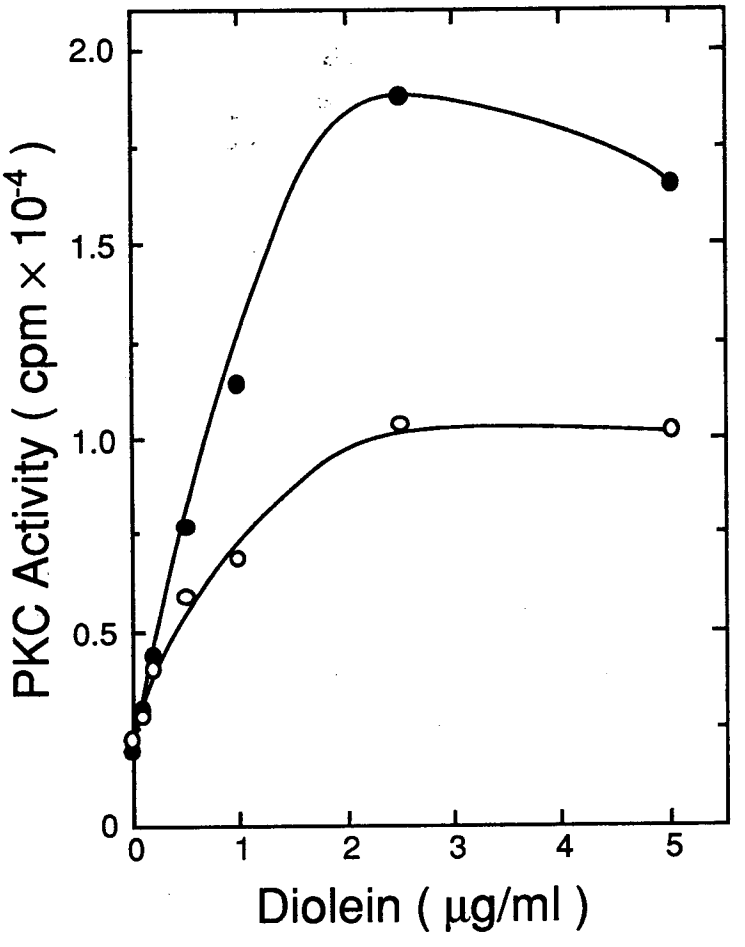

Fig. 16. Effect of lysoPC on the activation of PKC by DG.

The α-subspecies was assayed under the standard conditions except that various concentrations of diolein were added as indicated, with and without lysoPC. ●, 4-μM lysoPC; ○, no addition.

2–3 μg/ml (Fig. 16). It is noted that PKC showed only a little activity without diolein, both in the presence and absence of lysoPC. It is obvious, therefore, that lysoPC does not substitute for diolein. Fig. 16 also shows that lysoPC seems not to affect the sensitivity of the α-subspecies to diolein. Similar results were obtained for the β- and γ-subspecies (data not shown).

In the presence and absence of lysoPC, the activation of cPKC by various concentrations of PS was measured with DG (2.5 μg/ml) and $CaCl_2$ (1×10^{-6} M). In the presence of 4-μM lysoPC, the concentration of PS effective for the activation of the α-subspecies was lower than that observed in the absence of lysoPC (Fig. 17A). It is noted that PKC did not show any activity without PS, both in the presence and absence of lysoPC. It is evident, therefore, that lysoPC does not substitute for PS. The kinetic analysis for the concentration of PS revealed that lysoPC greatly increased the affinity of the enzyme for PS (Fig. 17B). Similar results were obtained for the β- and γ-subspecies (data not shown). Therefore, the enhancement of the PKC activity by lysoPC is mainly due to the increase in the affinity of PKC for PS.

III-5. Lysophospholipid specificity.

Acyl group specificity of lysoPC was examined for the enhancement of cPKC activity (Fig. 18). Palmitoyl (16:0, carbon chain length: number of double bond)-lysoPC was most effective among various molecular species lysoPC having different acyl moieties tested in this experiment. Caproyl (6:0)- and myristoyl (14:0)-lysoPC were less effective, and stearoyl (18:0)-lysoPC was ineffective. In the *sn*-1 position of PC, palmitoyl moiety is most abundant among the acyl groups of PC that occur in cell membranes (35). Thus, the activity of cPKC is enhanced by the most abundant lysoPC subtypes that may be produced from cell membranes. The polar group specificity of lysophospholipids was also investigated (Fig. 18). LysoPC was most effective for the enhancement of PKC

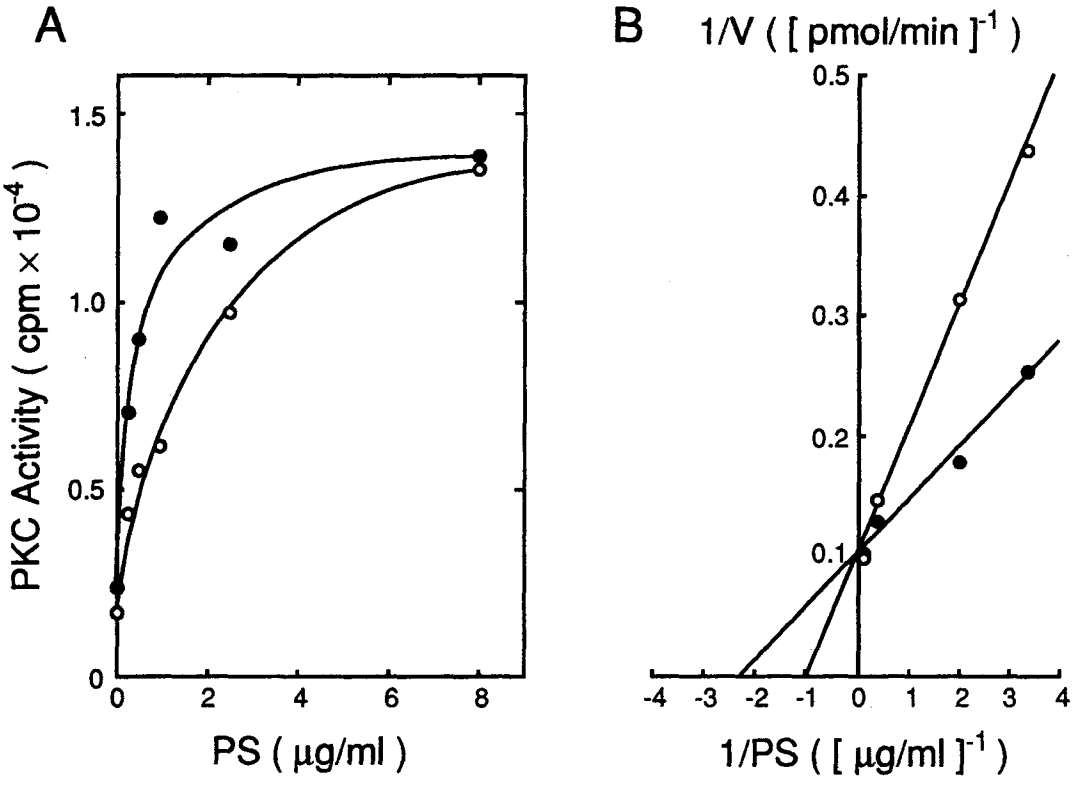

Fig. 17. Effect of lysoPC on the activation of PKC by PS.

The α-subspecies was assayed under the standard conditions described except that various concentrations of PS were added as indicated. A, Activation of cPKC by various concentrations of PS with and without lysoPC. ●, 4-μM lysoPC; ○, no addition. B, Double-reciprocal plots of data from (A). In the absence of lysoPC, Ka for PS = 1.1 μg/ml. In the presence of 4-μM lysoPC, Ka for PS = 0.44 μg/ml.

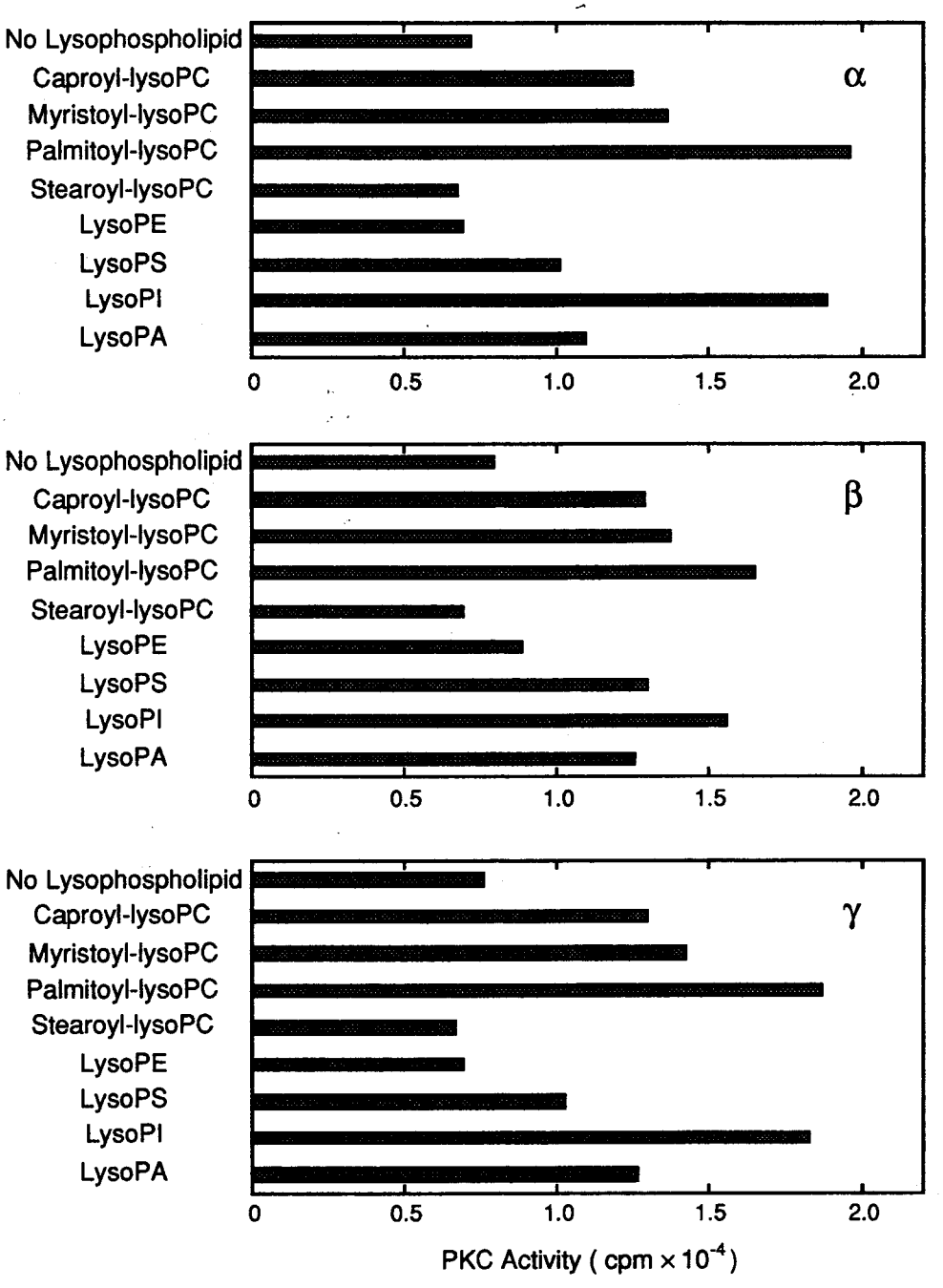

Fig. 18. Effect of various lysophospholipids on PKC activity.

Each PKC subspecies was assayed under the standard conditions described except that various lysophospholipids (4 μM) were added as indicated. α, the α-subspecies; β, the β-subspecies; γ, the γ-subspecies.

activity. LysoPI enhanced the activity to an extent similar to lysoPC. LysoPA and lysoPS were less effective and lysoPE was ineffective. There was no significant difference for lysophospholipid specificity among the α-, β-, and γ-subspecies (Fig. 18).

III-6. Phosphate acceptor proteins.

The effect of lysoPC on the phosphorylation of other substrate proteins was examined (Table 2). The phosphorylation of myelin basic protein (MBP) by the α-subspecies was enhanced at 4-μM lysoPC, and rather decreased at 32 μM. Similar result was obtained using myristoylated alanine-rich C kinase substrate (MARCKS, reviewed in (36)), one of the physiological substrate proteins of the PKC family. The phosphorylations by the β- and γ-subspecies were also enhanced under the same conditions. Therefore, lysoPC seems to show a dual action, stimulatory and inhibitory, on the cPKC activity for various substrate proteins.

Table 2. Effect of lysoPC on phosphorylation of various substrates by PKC.

LysoPC (μM)	Phosphorylation by PKC (%)		
	H1 histone	MBP	MARCKS
0	100	100	100
4	312	279	134
32	94	187	81

The α-subspecies was assayed as indicated under the standard conditions described in Materials & Methods except that various substrate proteins were used. Results are normalized to the phosphorylation of each substrate protein in the absence of lysoPC as 100%.

IV. Discussion

IV-1. Effect of lysoPC on the activity of PKC subspecies.

In Chapter 1, it was shown that lysoPC greatly potentiated T lymphocyte activation. Secretory group II PLA_2 was also shown to enhance T lymphocyte activation (37). This PLA_2 produces lysoPC by the hydrolysis of PC, and is found frequently in exudates at various sites of inflammation. In both cases, PLA_2 and lysoPC were ineffective without DG and Ca^{2+} ionophore, suggesting that lysoPC may interact with the PKC pathway. In Chapter 2, therefore, studies were undertaken to examine whether PKC may be one of the direct targets for the lysoPC action. An early report by Kuo and colleagues (38) have reported that lysoPC enhances the activity of an unfractionated mixture of PKC subspecies at higher concentrations of Ca^{2+}, whereas this lipid inhibits at lower concentrations of Ca^{2+} in a cell-free enzymatic system. In the present studies with the purified α-, β-, and γ-subspecies, lysoPC at low concentrations such as 4 μM always potentiates the DG-dependent activation of cPKC over a wide range of Ca^{2+} concentrations (Fig. 14). It seems possible that lysoPC enhances the cPKC activity at physiological concentrations of Ca^{2+}. Since lysoPC has a little effect, if any, on the δ-subspecies, and is rather inhibitory on the ε-subspecies (Fig. 15), it is plausible that lysoPC induces cellular responses, mainly through the activation of cPKC. In T lymphocytes, the α-, β-, δ-, ε-, ζ-, and η(L)-subspecies are shown to be expressed (39) A possibility of lysoPC action through the δ- and ε-subspecies *via* some other unknown mechanism may not be ruled out, however. The effects of lysoPC on the other two nPKCs (the η(L)- and θ-subspecies) and aPKCs (the ζ- and λ-subspecies) remain to be explored.

As to the mechanism of lysoPC to potentiate the cPKC activation, lysoPC does not appear to activate cPKC by itself, nor to substitute for DG or PS. Fig. 16 shows probably that lysoPC does not affect the sensitivity of cPKC to DG. Furthermore, lysoPC does not affect the phorbol-ester binding activity of cPKC

(data not shown). On the other hand, lysoPC greatly increases the affinity of cPKC for PS (Fig. 17). Therefore, it is likely that the potentiation of the cPKC activation by lysoPC is mainly due to increasing the affinity of cPKC for PS. However, it is also possible that lysoPC modulates a fine structure of physical states of mixed liposomes, such as shape, fluidity, miscibility, and stability. The precise interaction among cPKC, substrate, and liposomes still remains unclear. LysoPC enhances the phosphorylation of all substrates tested, although there was some kinetic variation (Table 2).

cis-Unsaturated fatty acids such as arachidonic acid (20:4) and linoleic acid (18:2), the other products of PC hydrolysis by PLA_2, also enhance the activity of PKC in the presence of PS and DG (25). However, *cis*-unsaturated fatty acids do not increase an affinity of the enzyme for PS. *cis*-Unsaturated fatty acids increase Ca^{2+}-sensitivity of the enzyme for activation, and this lipids augment PKC activity to the maximum at physiologically low concentrations. On the other hand, lysoPC does not increase Ca^{2+}-sensitivity. The activities of the α-, β-, γ-, and ε-subspecies are all increased by *cis*-unsaturated fatty acids (11, 25), whereas, lysoPC enhances only the activity of cPKC, and rather inhibits the ε-subspecies. This difference of the actions of these two products of PC hydrolysis by PLA_2 on the sensitivity of PKC to Ca^{2+} and PS also remains to be clarified.

IV-2. Comparison between the effects of lysoPC on T lymphocytes and on the activity of cPKC.

In Chapter 1, it is shown that lysoPC does not affect the sensitivity of T lymphocyte activation to membrane-permeant DG nor intracellular Ca^{2+}. In a cell-free enzymatic system, lysoPC also does not affect the sensitivity of cPKC activity to DG nor Ca^{2+}. Thus, the enhancement of cPKC activity by lysoPC seems to have similar property for DG and Ca^{2+}-sensitivity, to the potentiation of T lymphocyte activation. However, it is unclear whether, in T lymphocyte, lysoPC

enhances the association of cPKC with PS of cell membranes, although, in cell-free system, lysoPC increases the affinity of cPKC for PS.

The concentration of lysoPC effective for cell-free activation of cPKC is different from that observed for T lymphocyte activation. In a cell-free system, the optimum concentration of lysoPC is 4 μM, whereas the optimum concentration for T lymphocyte activation is 50–100 μM as described in Chapter 1. It is possible that the serum in culture medium for T lymphocytes traps lysoPC, and prevents the cells from quick uptake of this lipid. However, it remains to be determined the actual concentration of lysoPC which is taken up from culture medium, and also the concentration of this lysophospholipid which may be endogenously produced after cell stimulation by physiological signals. For both the *in vitro* assay system and the *in vivo* potentiation of T lymphocyte activation, lysoPC was most effective among various lysophospholipids tested. However, the spectra of the effective lysophospholipids in both systems are slightly different. In the cell-free system, lysoPI, lysoPS, and lysoPA were effective to some extents (Fig. 18), but these lysophospholipids were practically ineffective when added exogenously to T lymphocytes (Fig. 6). This difference may be due to the efficiency of the uptake of lysophospholipids into the cell, and the rate of the degradation or metabolism of the lysophospholipids within the cell.

In Chapter 1, it was shown that in T lymphocytes, lysoPC may accumulate in a time-dependent manner upon a physiological stimulation, such as that of T lymphocyte receptor by antigenic signal (Fig. 10). Furthermore, the secretory group II PLA_2, at the concentrations observed in inflammatory sites, may induce sustained formation of lysoPC in T lymphocytes which are activated by the simultaneous presence of a membrane-permeant DG and Ca^{2+} ionophore (37). These results suggest that the lysoPC level in stimulated cells may be elevated for prolonged period of time. Therefore, lysoPC generated from PC hydrolysis by PLA_2 as well as DG generated from PC through PLD pathway may prolong and sustain the activation of PKC, resulting in the long-term cellular responses such

as cell proliferation and differentiation. However, further investigation is needed to elucidate the precise biochemical mechanism of the stimulatory action of lysoPC on cell activation.

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Implications & Perspectives

In Chapter 1, it is proposed that PLA_2 may be directly involved in long-term cellular responses such as T lymphocyte activation probably through the action of lysoPC, one of the products of PC hydrolysis by PLA_2, which may potentiate the PKC pathway. In Chapter 2, lysoPC is capable of enhancing the activity of cPKC *in vitro* at physiological concentrations of Ca^{2+}, and this enhancement depends on DG. LysoPC appears, therefore, to act as a messenger molecule to elicit cellular responses from aspects described below. 1, LysoPC accumulates within the cell, in response to cell surface receptor stimulation (stimulation of TCR by antigenic signal) which induces cellular responses (T lymphocyte activation). 2, When added to the cell, lysoPC can enhance the cellular responses (enhancement of T lymphocyte activation). 3, LysoPC may activate its target (cPKC) in some cell-free systems. However, since DG is absolutely required for the lysoPC action as noted above, it acts as an enhancer or modulator molecule rather than a second messenger in cell signaling.

The effect of lysoPC on cell activation and its possible target protein *in vitro* are investigated in this study. However, it is still unclear whether within intact cells, lysoPC indeed activates cPKC. Two contradictory results have been reported (1, 2). When ET-18-OCH_3, one of derivatives of alkyllysoPC, was added to human promyelocytic leukemia cells (HL-60), the activity of PKC in the cells was inhibited or stimulated. Unlike mitogen-activated protein kinases (MAP kinases) which are activated by phosphorylation [reviewed in (3, 4)], it is difficult to examine the activation state of PKC isolated from the cells. It had been thought that the translocation of PKC from cytosol to membranes is an indicator of the activation state of PKC, since the translocation is induced by the application of phorbol esters to the cell. However, it has been described that the translocation of PKC appears not to be observed in a later-phase of DG

production, responding to thyrotropin-releasing hormone (TRH), in rat pituitary GH_3 cells (5). Nevertheless this hormone induces long-term cellular responses such as prolactin synthesis. Thus, the translocation of PKC may not always serve as an indicator of the activation of PKC in response to physiological signals. The degree of phosphorylation of MARCKS protein is frequently used as an indicator of the activation of PKC in the cell. However, since the phosphorylation of MARCKS protein is observed only transiently (6), this phosphorylation state is not able to be used as the indicator of sustained activation of PKC. It is known that stathmin (alias: prosolin), which is a ubiquitous, phylogenetically conserved protein present in the cytoplasm, is phosphorylated by the application of phorbol esters [reviewed in (7)], and this phosphorylation is sustained by various physiological signals such as antigenic signals of T lymphocytes (8) and nerve growth factor (NGF) in rat pheochromocytoma PC12 cells (9). It may be possible that the phosphorylation state of stathmin is able to used as a better indicator of activation state of PKC when lysoPC is added to T lymphocyte.

Phospholipids had been once thought to be simply constituent molecules of cell membranes. However, since the discovery that DG and IP_3, the metabolites of PIP_2-hydrolysis by PLC, are directly involved in intracellular signal transduction, our knowledges of the mechanism of cell signaling have been expanded rapidly, and various phospholipases and their metabolites are clarified to be involved not only in short-term cellular responses but also in long-term cellular responses (10-12). PLC evokes a transient elevation of intracellular Ca^{2+} and DG, and is able to induce short-term cellular responses as well as to trigger long-term cellular responses. It is also becoming clearer that the DG production from PLD activation may be sustained, and is proposed to prolong PKC activation to cause long-term cellular responses. *cis*-Unsaturated fatty acids, one of the products of PC hydrolysis by PLA_2, are shown to potentiate cellular responses that are caused by PKC activation, such as platelet activation

(13). LysoPC, the other products of PC hydrolysis by PLA_2, is shown to potentiate long-term cellular responses such as T lymphocyte activation, as described in this study. Schütze *et al.* have reported very recently that sphingomyelin, which is a phospholipid having a sphingosine backbone instead of glycerol, is degradated responding to physiological signal such as tumor necrosis factor (TNF) (14). They have postulated that the long-term cellular responses induced by TNF may be mediated through the PKC pathway that is activated by DG from PC hydrolysis by PC-specific PLC, and that another signal transduction pathway may occur in the response to the ceramide production from sphingomyelin hydrolysis by sphingomyelinase. Thus, various cell signaling pathways including several phospholipases and their metabolites may play roles in cellular responses such as proliferation and differentiation. Although it is still far from full understanding the whole picture of such cellular processes, the present studies may hopefully be one of the step to approach this problem.

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Chapter 1.

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