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Potentiation of diacylglycerol-induced activation of protein kinase C by lysophospholipids

Subspecies difference*

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Lysophospholipid, particularly 2-lysophosphatidylcholine (lysoPtdCho), significantly potentiates the diacylglycerol (DAG)-induced activation of protein kinase C (PKC) in vitro. LysoPtdCho shows no effect, unless DAG and phosphatidylserine (PtdSer) are present. This lysoPtdCho action also depends on its own as well as on Ca^{2+} concentration. At physiological Ca^{2+} concentrations, the activation of the α-, β-, and γ-subspecies (cPKC) is enhanced by lysoPtdCho in the 10^{-6} M range, but inversely inhibited in the 10^{-5} M range. The δ- and ε-subspecies (nPKC), which are enzymatically insensitive to Ca^{2+}, are mostly inhibited by lysoPtdCho at its low concentrations. The enhancement of cPKC activation by lysoPtdCho is due to the increase in an apparent affinity of the enzyme for PtdSer but not for DAG. The results may account, at least in part, for the previous observations made with intact cell systems that lysoPtdCho significantly potentiates the DAG-induced cellular responses such as T-lymphocyte activation and HL-60 cell differentiation [(1992) Trends Biochem. Sci. 17, 414–417].

Protein kinase C; Lysophosphatidylcholine; Phospholipase A_2

1. INTRODUCTION

2-Lysophosphatidylcholine (lysoPtdCho), one of the products of phosphatidylcholine (PtdCho) hydrolysis by phospholipase A_2, greatly potentiates the cellular responses such as T-lymphocyte activation and HL-60 cell differentiation, that can be induced by a membrane-permeant diacylglycerol (DAG) or tumour-promoting phorbol esters [1,2]. Several *cis*-unsaturated fatty acids, such as oleic, linoleic, and linolenic acids, the other products of PtdCho hydrolysis by phospholipase A_2, are also shown to enhance the DAG-induced activation of protein kinase C (PKC) in vitro [3] as well as the DAG-induced cellular responses such as platelet release reaction [4]. In 1988, based on the observation that lysophospholipid, particularly lysoPtdCho, stimulates PKC (an unfractionated mixture of several PKC subspecies) at its lower concentrations but conversely inhibits the enzyme at its higher concentrations, Oishi et al. [5] have postulated a role of this lysophospholipid in the regulation of PKC activation.

Ten subspecies of PKC have been subsequently identified from mammalian tissues, which may be divided into three subgroups [6]. cPKC consists of the α-, β_I-, β_{II}-, and γ-subspecies (the β_I and β_{II} are enzymologically indistinguishable from each other [7]), nPKC consists of the δ-, ε-, η(L)-, and θ-subspecies, and aPKC consists of the ζ-, and λ-subspecies, although the η(L)-, θ-, and λ-subspecies have not yet been isolated from tissues [6]. Extending the studies by Oishi et al. [5], this report will describe the mode of lysoPtdCho action on the individual PKC subspecies, in an attempt to explore the biochemical basis of the enhancement of the DAG-induced cellular responses by lysoPtdCho.

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Abbreviations: lysoPtdCho, 2-lysophosphatidylcholine; PtdCho, phosphatidylcholine; DAG, diacylglycerol; PKC, protein kinase C; cPKC, classical or conventional PKC; nPKC, new PKC; aPKC, atypical PKC; MARCKS, myristoylated alanine-rich C kinase substrate; MBP, myelin basic protein; PtdSer, phosphatidylserine.

*The data are taken in part from the dissertation that has been submitted by Y. Sasaki to the Graduate School of Science and Technology, Kobe University, in partial fulfillment of the requirement for doctoral degree.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was a product of New England Nuclear. Calf thymus H1 histone was prepared as described [8]. Myristoylated alanine-rich C kinase substrate (MARCKS) was prepared from bovine brain by the method of Graff et al. [9]. Bovine myelin basic protein (MBP), L-α-oleoyl-lysophosphatidic acid, L-α-lysoPtdCho (bovine brain), L-α-lysophosphatidylethanolamine (egg yolk), L-α-lysophosphatidylinositol (soy bean), L-α-lysophosphatidyl-L-serine (bovine brain), L-α-

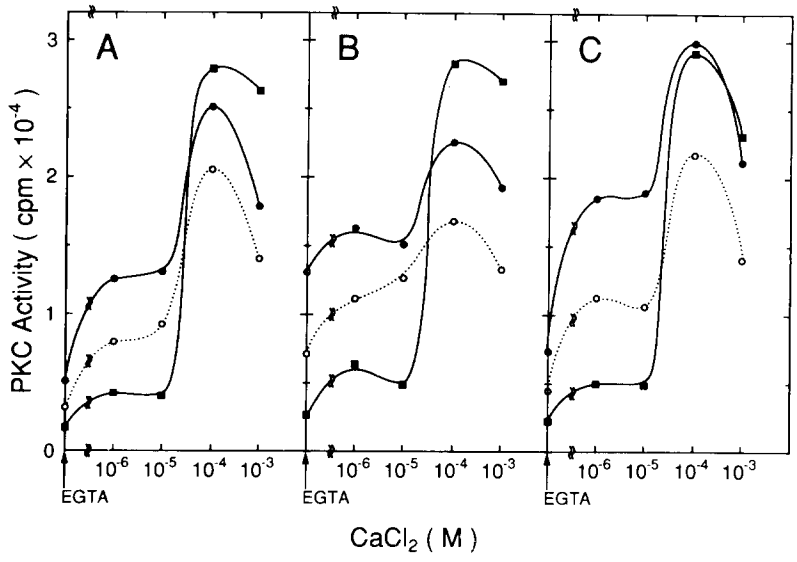

Fig. 1. Effect of lysoPtdCho on cPKC activity. Each cPKC subspecies (≈0.05 unit) was assayed with H1 histone as a model substrate under the standard conditions except that various concentrations of $CaCl_2$ were added in the presence (●—●, 4 μM; ■—■, 16 μM) or absence (○—○) of lysoPtdCho. EGTA (5 mM) instead of $CaCl_2$ was added to the reaction mixture where indicated by arrows. (A) The α-subspecies; (B) the β-subspecies; (C) the γ-subspecies.

caproyl-lysoPtdCho, L-α-myristoyl-lysoPtdCho, L-α-palmitoyl-lysoPtdCho, and L-α-stearoyl-lysoPtdCho were obtained from Sigma. Phosphatidylserine (PtdSer, bovine brain) and 1,2-*sn*-diolein were purchased from Serdary Research Laboratories (London, Ont., Canada). Other chemicals were purchased from commercial sources.

2.2. *PKC and assay*

The α-, β-, γ-, δ-, and ε-subspecies were purified from rat brain as described [10–12]. All preparations used were practically pure. The enzyme activity was assayed as described [13] with slight modifications. The standard reaction mixture (0.25 ml) contained 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 1 μM $CaCl_2$, 10 μM [γ-^{32}P]ATP (150–250 cpm/pmol for the α-, β-, and γ-subspecies; and 500–700 cpm/pmol for the δ- and ε-subspecies), 50 μg of calf thymus H1 histone, the enzyme, and liposomes composed of PtdSer (1 μg/ml), diolein (2.5 μg/ml), and L-α-palmitoyl-lysoPtdCho (4 μM) unless otherwise indicated in each experiment. The liposomes were prepared as follows. PtdSer, diolein, and lysophospholipid were mixed first in chloroform, and dried under a nitrogen stream. The residue was then sonicated for 3 min in 20 mM Tris-HCl (pH 7.5), and added directly to the reaction mixture. The reaction was started by the addition of enzyme. After incubation for 5 min (for the α-, β-, and γ-subspecies) or 10 min (for the δ- and ε-subspecies) at 30°C, the reaction was stopped by the addition of 25% trichloroacetic acid, and acid-precipitable materials were collected on a nitrocellulose filter. The radioactivity was determined 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 [13]. One unit of nPKC, the δ- and ε-subspecies, was defined as above, except that MBP was employed as phosphate acceptor [11,12].

2.3. *Other methods*

SDS-PAGE and protein determination were made as described [14,15]. The phosphorylation of MARCKS by PKC was performed as described above, and the incorporation of the radioactivity into MARCKS was quantified after gel electrophoresis with a BAS-2000 Bioimage analyzer (Fuji).

3. RESULTS

3.1. *LysoPtdCho effect on cPKC activation*

The members of cPKC subgroup were assayed in the presence of a relatively low (4 μM) or high (16 μM) concentration of lysoPtdCho at various Ca^{2+} concentrations (Fig. 1). LysoPtdCho at higher concentrations showed a biphasic, stimulatory and inhibitory, action on each subspecies of cPKC, depending on the Ca^{2+} concentration. It was noted, however, that lysoPtdCho at lower concentrations was always stimulatory, irrespective of the Ca^{2+} concentrations. LysoPtdCho alone was inactive, and PtdSer and DAG were essential for this PKC activation.

The maximum stimulatory effect of lysoPtdCho varied with the Ca^{2+} concentration (Fig. 2). At physiological concentrations of Ca^{2+}, lysoPtdCho stimulated this PKC activation in its micromolar range, whereas at unusually high concentrations of Ca^{2+}, more lysoPtdCho was needed for its maximum action. Several *cis*-unsaturated fatty acids such as oleic and linoleic acids greatly enhanced the activation of cPKC enzymes synergistically with DAG, particularly at lower concentrations of Ca^{2+}, as described [3]. The simultaneous presence of *cis*-unsaturated fatty acids and lysoPtdCho greatly enhanced the cPKC activation at less than the micromolar concentrations of Ca^{2+}. The enhancement of this PKC activation by lysoPtdCho described herein was not synergistic to the action of *cis*-unsaturated fatty acids. Essentially similar results were obtained for the α-, β-, and γ-subspecies.

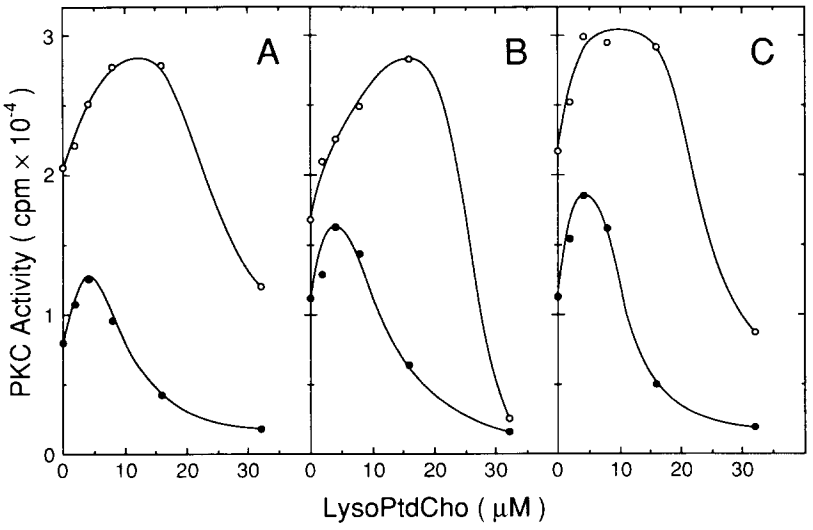

Fig. 2. Effect of lysoPtdCho concentration on cPKC activity. Each cPKC subspecies (≈0.05 unit) was assayed with H1 histone as a model substrate under the standard conditions except that various concentrations of lysoPtdCho were added in the presence of $CaCl_2$ at 1×10^{-6} M (●) or 1×10^{-4} M (○). (A) The α-subspecies; (B) the β-subspecies; (C) the γ-subspecies.

3.2. *LysoPtdCho effect on nPKC activation*

In contrast to cPKC, nPKC enzymes, which lack the C2 region and do not require Ca^{2+} for their activation [6], are markedly inhibited by lysoPtdCho over a wide range of its concentrations (Figs. 3 and 4), although the activation of the δ-subspecies was slightly enhanced by lysoPtdCho at low concentrations (Figs. 3A and 4A).

3.3. *Kinetic analysis of lysoPtdCho action*

The enhancement of cPKC activation by lysoPtdCho (4 μM) was measured at various concentrations of PtdSer and a fixed concentration of DAG (2.5 μg/ml) and Ca^{2+} (1 μM) (Fig. 5). The double reciprocal plot revealed that an apparent affinity of the enzyme for PtdSer was increased by the addition of lysoPtdCho.

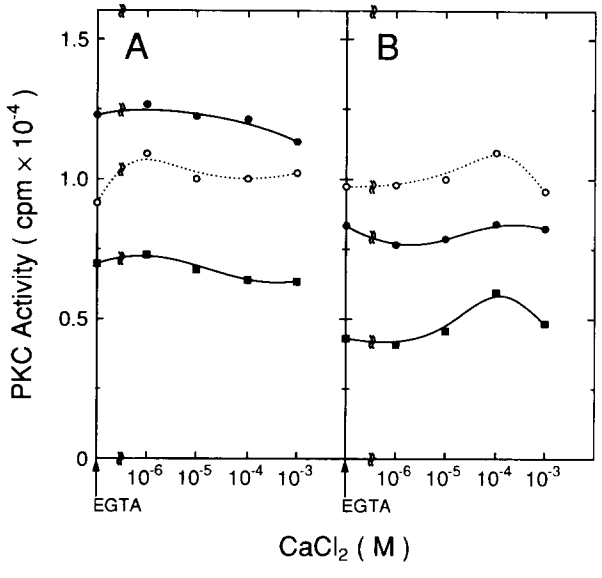

Fig. 3. Effect of lysoPtdCho on nPKC activity. Each nPKC subspecies (≈0.002 unit) was assayed with H1 histone as a model substrate under the standard conditions except that various concentrations of $CaCl_2$ were added in the presence (●—●, 4 μM; ■—■, 16 μM) or absence (○—○) of lysoPtdCho. EGTA (5 mM) instead of $CaCl_2$ was added to the reaction mixture where indicated by arrows. (A) The δ-subspecies; (B) the ε-subspecies.

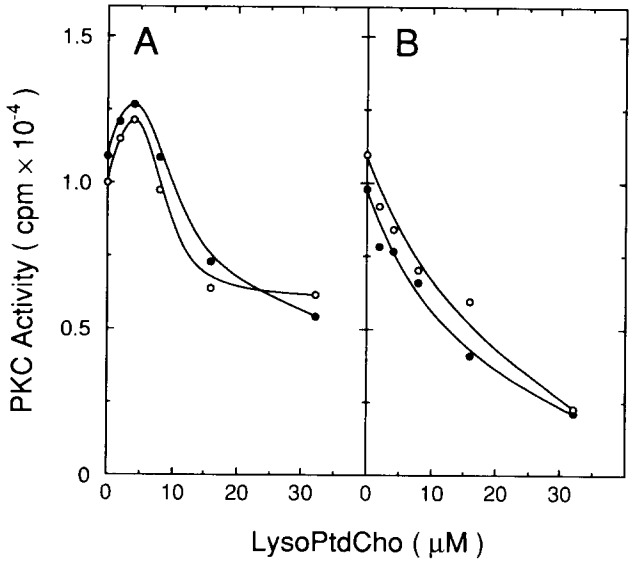

Fig. 4. Effect of lysoPtdCho concentration on nPKC activity. Each nPKC subspecies (≈0.002 unit) was assayed with H1 histone as a model substrate under the standard conditions except that various concentrations of lysoPtdCho were added in the presence of $CaCl_2$ at 1×10^{-6} M (●) or 1×10^{-4} M (○). (A) The δ-subspecies; (B) the ε-subspecies.

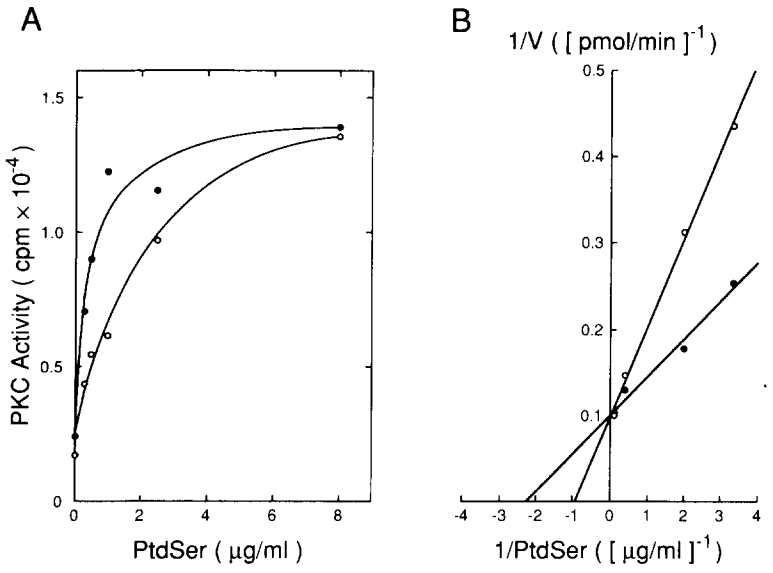

Fig. 5. Effect of PtdSer concentration on cPKC activation by lysoPtdCho. (A) The α-subspecies (≈0.05 unit) was assayed with H1 histone as a model substrate under the standard conditions except that various concentrations of PtdSer were added in the presence (●, 4 μM) or absence (○) of lysoPtdCho. (B) The Lineweaver-Burk plot of the results given in (A).

LysoPtdCho was inactive unless PtdSer was present, indicating that lysoPtdCho did not substitute for PtdSer, which was absolutely required for PKC activation. Similar results were obtained for the α-, β-, and γ-subspecies.

Next, the activation of cPKC by various concentrations of DAG at a fixed amount of PtdSer, Ca^{2+}, and lysoPtdCho was examined. The results indicated that lysoPtdCho did not cause any significant effect on the affinity of the enzymes for DAG.

3.4. *Specificity of lysophospholipid*

Among various lysophospholipids tested, lysoPtdCho and lysophosphatidylinositol were most active to enhance the cPKC activation (Fig. 6). Lysophosphatidylserine and lysophosphatidic acid were less active, and lysophosphatidylethanolamine was practically inactive.

Several molecular species of lysoPtdCho having various fatty acyl moieties at the position 1 were tested. Although many of the lysoPtdCho species were active to various extents, palmitoyl-lysoPtdCho was most effective. Essentially similar results were obtained for the α-, β-, and γ-subspecies.

3.5. *Phosphate acceptor proteins*

The lipid requirement of PKC activation frequently varies with the phosphate acceptor proteins [16]. The effect of lysoPtdCho on the cPKC activation described above was observed also for other proteins such as MBP and MARCKS as phosphate acceptors (Table I). The MARCKS protein is a physiological substrate of PKC which occurs in many tissues and cell types [9].

4. DISCUSSION

Extending the previous observation made by Kuo and his coworkers [5] who employed an unfractionated mixture of several subspecies of PKC, the present studies show that lysoPtdCho and Ca^{2+} at their low concentrations, presumably under physiological conditions, may potentiate the activation of the α-, β-, and γ-subspecies but not other subspecies of PKC, although the action of lysoPtdCho on aPKC, the ζ- and λ-subspecies, remains unknown. The enzymes of aPKC subgroup are not activated by DAG nor by phorbol esters, and their biological roles remain to be explored [6].

Preceding reports from this laboratory [1,2] have shown that lysoPtdCho greatly potentiates cellular responses, such as T-lymphocyte activation and HL-60 cell differentiation, that are induced by a membrane-

Table I

Phosphorylation of various PKC substrates in the presence of lysoPtdCho

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

The α-subspecies (≈0.05 unit) was assayed under the standard conditions except that various proteins were used as phosphate acceptors. The radioactivity incorporated into each substrate was determined as described. The values presented are normalized to the phosphorylation of each substrate protein in the absence of lysoPtdCho as 100%.

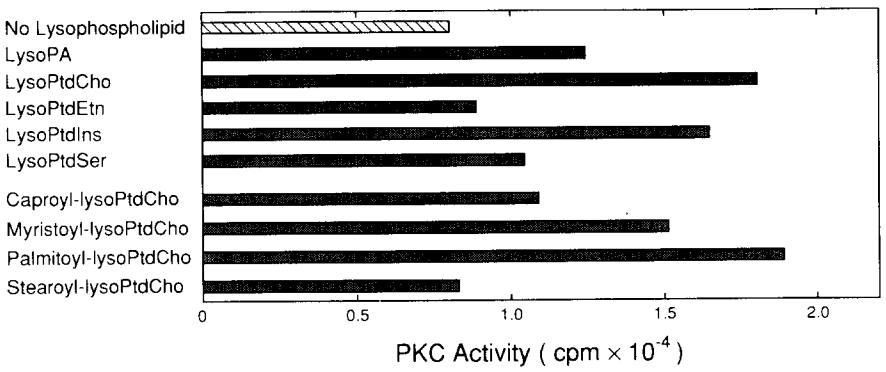

Fig. 6. Effect of various lysophospholipids on the activity of cPKC. The α-subspecies was assayed under the standard conditions except that various lysophospholipids (4 μM) were added as indicated. LysoPA, lysophosphatidic acid; LysoPtdEtn, lysophosphatidylethanolamine; LysoPtdIns, lysophosphatidylinositol; LysoPtdSer, lysophosphatidylserine.

permeant DAG or a tumour-promoting phorbol ester, suggesting that lysoPtdCho can enhance the DAG-induced PKC activation. Other lysophospholipids are apparently inactive. However, the present results described above show that the enzyme activation in vitro can be enhanced by several lysophospholipids, including lysoPtdCho and lysophosphatidylinositol. The latter lysophospholipid is inactive in intact cell systems [1,2]. The reason for this discrepancy of the specificities of lysophospholipids in in vivo and in vitro systems is unclear, but it may be partly due to the difference of their metabolic rates within the cell. It also remains to be explored whether the enhancement of cPKC activation described herein indeed accounts for the stimulatory action of lysoPtdCho on the cellular responses observed in intact cell systems. The precise biochemical mechanism of PKC activation by combinations of several lipid components described in this paper is an important problem to be clarified.

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