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# Potential role of phospholipase $A_2$ in HL-60 cell differentiation to macrophages induced by protein kinase C activation

**(2-lysophosphatidylcholine/cis-unsaturated fatty acid/phorbol ester/diacylglycerol)**

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**ABSTRACT** **2-Lysophosphatidylcholine and cis-unsaturated fatty acids such as linoleic and linolenic acids, which are the products of the hydrolysis of phosphatidylcholine catalyzed by phospholipase $A_2$ (EC 3.1.1.4), significantly potentiate the differentiation of HL-60 cells to macrophages that is induced by either a membrane-permeant diacylglycerol or a phorbol ester. The cell differentiation was assayed by measuring the expression of CD11b, one of the cell surface markers of macrophages, and also by the appearance of phagocytic activity. Snake venom phospholipase $A_2$ added directly to the cells is also active for this potentiation. Neither lysophosphatidylcholine, fatty acid, nor phospholipase $A_2$ is active unless a membrane-permeant diacylglycerol or a phorbol ester is present. The results presented provide further evidence that activation of phospholipase $A_2$ may be intimately related to the signal transduction pathway through protein kinase C.**

The HL-60 cell line is frequently used as a model system to investigate the mechanism of cell differentiation, since retinoic acid and several other chemicals lead the cells to produce granulocytes, whereas phorbol 12-myristate 13-acetate (PMA) generates macrophages from these cells (for review, see ref. 1). A single dose of PMA can induce the differentiation of HL-60 cells to macrophages. On the other hand, a single dose of a membrane-permeant diacylglycerol (DAG), 1,2-dioctanoylglycerol (1,2-$DiC_8$), is normally insufficient to induce this differentiation because it is rapidly metabolized and disappears from the cells (2–5). Repeated additions of 1,2-$DiC_8$, however, can induce the cell differentiation, as measured by the expression of CD11b, a cell surface marker of macrophages (5), suggesting that sustained activation of protein kinase C (PKC) is essential for causing this cellular response (6, 7). The formation of DAG from receptor-mediated hydrolysis of inositol phospholipids is usually transient, and recent evidence suggests that, at a later phase in cellular responses, DAG is produced from the signal-induced hydrolysis of phosphatidylcholine (PtdCho), and this reaction is initiated presumably by phospholipase D activation (for reviews, see refs. 8–11).

We have previously reported (12, 13) that 2-lysophosphatidylcholine (lysoPtdCho) and cis-unsaturated fatty acids, both of which can be produced from PtdCho hydrolysis by phospholipase $A_2$ (EC 3.1.1.4), significantly enhance T-lymphocyte activation and platelet release reaction, which are induced by 1,2-$DiC_8$ and PMA. The hydrolysis of various membrane phospholipids, therefore, appears to play roles in eliciting short-term as well as long-term cellular responses such as release reaction and cell proliferation. The present studies were undertaken, with HL-60 cells as an experimental system, to demonstrate further that phospholipase $A_2$ activation may intensify the role of PKC in signal transduction, eventually leading to cell differentiation.

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## MATERIALS AND METHODS

**Materials.** HL-60 cells donated by J. Minowada (Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama, Japan) were maintained at a cell density between 0.1 and 1.0 × $10^6$ cells per ml as a suspension in RPMI 1640 medium supplemented with 5% fetal bovine serum (Flow Laboratories) at 37°C in a humidified 5% $CO_2$ atmosphere.

**Chemicals.** A membrane-permeant DAG, 1,2-$DiC_8$, was obtained from Nacalai Tesque (Kyoto). This preparation consists of approximately 95% DL-1,2-$DiC_8$ and 5% 1,3-$DiC_8$ as estimated by thin-layer chromatography. PMA was a product of LC Services (Woburn, MA). Lysophospholipids, fatty acids, and *Crotalus adamanteus* phospholipase $A_2$ were obtained from Sigma. Phospholipase $A_2$ activity was determined by assaying with dipalmitoylphosphatidylcholine as a substrate. One unit of phospholipase $A_2$ was defined as the amount of enzyme that released 1 nmol of palmitic acid per min. Other chemicals were obtained from commercial sources.

**Determination of CD11b Expression.** HL-60 cells were stimulated and incubated as specified in each experiment. After incubation, the cells were collected, washed, resuspended in phosphate-buffered saline (PBS) containing 20% human serum at a density of 4.0 × $10^6$ cells per ml, and allowed to stand for 30 min at 4°C to saturate Fc binding sites. After three washes, the cells (4.0 × $10^6$ cells per ml) were treated for 30 min at 4°C with an appropriately diluted mouse monoclonal antibody against CD11b (Immunotech, Marseille, France) or with mouse IgG as a control. After three washes, the cells (4.0 × $10^6$ cells per ml) were incubated for 30 min at 4°C with a fluorescein-conjugated goat affinity-purified $F(ab')_2$ fragment that recognizes mouse IgG (Cappel Laboratories) and then washed and suspended in PBS. The fluorescence was determined by analysis of 5000 cells with a flow cytometer (Cyto ACE-150, Japan Spectroscopic, Tokyo).

**Assay of Phagocytosis.** The phagocytic activity was determined by measuring the uptake of fluorescent microspheres (Fluoresbrite Carboxylate Microspheres, 1.75 μm in diameter, Polysciences), as described by Blair *et al.* (14). The cells (2.0 × $10^5$ per ml) were stimulated as specified in each experiment in the presence of 5.0 × $10^5$ fluorescent microspheres per ml. After a 24-hr incubation, cells were washed, and fluorescent intensity was determined with the flow cytometer.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; $DiC_8$, dioctanoylglycerol; PKC, protein kinase C; PtdCho, phosphatidylcholine; lysoPtdCho, 2-lysophosphatidylcholine.

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## RESULTS

**Effect of LysoPtdCho and Linoleic Acid on CD11b Expression.** Although a single dose of phorbol ester can induce the expression of CD11b on the HL-60 cell surface, a single dose of membrane-permeant DAG is normally insufficient to cause this differentiation, because the cells metabolize the exogenously added DAG very rapidly. Thus, the extent of CD11b expression was inversely related to the cell density in the medium and roughly proportional to the amount of 1,2-$DiC_8$ added as described (5).

In the experiments shown in Fig. 1, 1,2-$DiC_8$ (50 $\mu$M) was added to a suspension of HL-60 cells ($1.0 \times 10^5$ cells per ml) three times with 3-hr intervals. Forty-eight hours after the first treatment, cell differentiation to macrophages was determined by measuring the expression of CD11b on the cell surface. These conditions can be directly comparable to those used in our earlier experiments (5). This amount and interval of 1,2-$DiC_8$ additions were still insufficient to cause full expression of the cell surface marker (Fig. 1*H*). A control experiment without any addition is given in Fig. 1*G*. Supplement of either lysoPtdCho or linoleic acid to the membrane-permeant DAG significantly enhanced the differentiation (Fig. 1 *I* and *J*, respectively), although lysoPtdCho or linoleic acid alone was practically inactive unless DAG was added (Fig. 1 *K* and *L*, respectively). In contrast to 1,2-$DiC_8$, large portions of lysoPtdCho and linoleic acid added were trapped by the serum proteins and remained in the medium during the incubation. These lipids, therefore, were added to the medium only once at the beginning of incubation. The effects of lysoPtdCho and fatty acid were both dose-dependent but not synergistic.

Similar results were obtained when PMA was used at submaximal concentrations instead of 1,2-$DiC_8$ as shown in Fig. 2. Direct addition of the snake venom phospholipase $A_2$ was also effective in the enhancement of the PMA-induced CD11b expression (Fig. 2*I*).

**Effect of LysoPtdCho and Linoleic Acid on Phagocytic Activity.** Phagocytic activity, another marker of HL-60 cell differentiation to macrophage, was also enhanced by the addition of lysoPtdCho or linoleic acid to the PMA-stimulated HL-60 cells, as shown in Fig. 3. The effects of lysoPtdCho

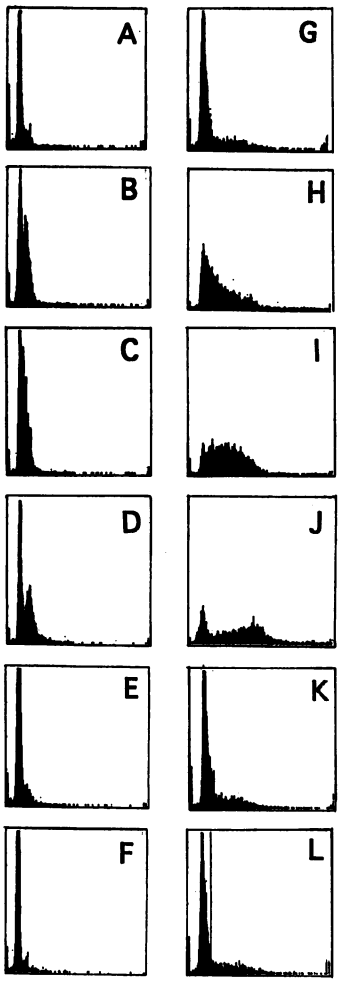


FIG. 1. Stimulatory actions of lysoPtdCho and linoleic acid on CD11b expression induced by repeated additions of 1,2-$DiC_8$. HL-60 cells ($1.0 \times 10^5$ cells per ml) were treated three times with 1,2-$DiC_8$ (50 $\mu$M) at 3-hr intervals in the presence of lysoPtdCho (50 $\mu$M, *C* and *I*) or linoleic acid (50 $\mu$M, *D* and *J*). As control experiments, the cells were incubated with repeated additions of 1,2-$DiC_8$ alone (*B* and *H*), with lysoPtdCho alone (*E* and *K*), with linoleic acid alone (*F* and *L*), or without any additions (*A* and *G*). After a 48-hr incubation, the cells were stained by the indirect immunofluorostain procedure either with a monoclonal antibody against CD11b (*G–L*) as the first antibody or with nonimmune mouse IgG as a control (*A–F*). Then the fluorescent intensity was determined. The fluorescent intensity (logarithmic scale) is represented on the horizontal axis, and the cell number is on the vertical axis.

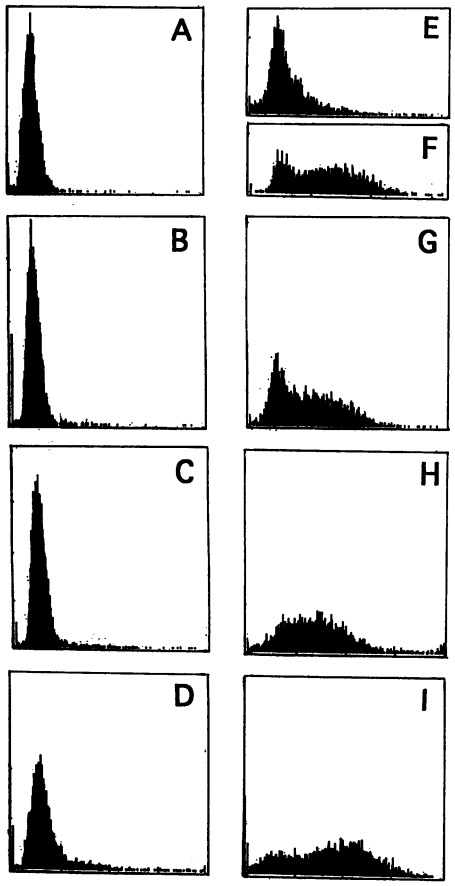


FIG. 2. Stimulatory actions of lysoPtdCho, linoleic acid, and phospholipase $A_2$ on CD11b expression induced by a single addition of PMA. HL-60 cells ($2.0 \times 10^5$ cells per ml) were treated with 1 nM PMA in the presence of lysoPtdCho (50 $\mu$M, *B* and *G*), linoleic acid (50 $\mu$M, *C* and *H*), or *C. adamanteus* phospholipase $A_2$ (50 units/ml, *D* and *I*). As control experiments, the cells were incubated with PMA alone (1 nM, *A* and *E*; 100 nM, *F*). After a 24-hr incubation, the cells were stained by the indirect immunofluorostain procedure either with a monoclonal antibody against CD11b as the first antibody (*E–I*) or with nonimmune mouse IgG as a control (*A–D*). Then the fluorescent intensity was determined. The fluorescent intensity (logarithmic scale) is represented on the horizontal axis, and the cell number is on the vertical axis.

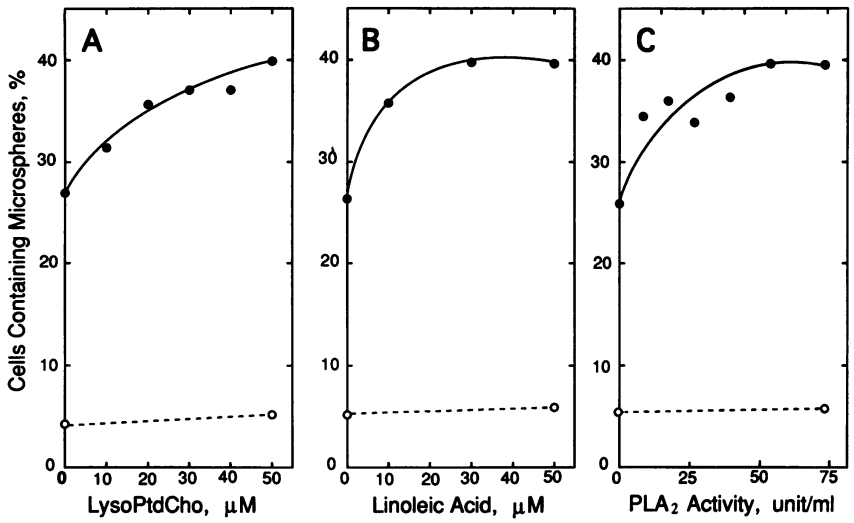


FIG. 3. Phagocytic activity of PMA-stimulated HL-60 cells incubated with lysoPtdCho, linoleic acid, or snake venom phospholipase $A_2$. HL-60 cells ($2.0 \times 10^5$ cells per ml) were incubated with various amounts of lysoPtdCho (*A*), linoleic acid (*B*), or *C. adamanteus* venom phospholipase $A_2$ (*C*) in the presence (●——●) or absence (○- - -○) of 5 nM PMA. After a 24-hr incubation, phagocytic activity was determined as described.

and linoleic acid were again dose-dependent up to ≈50 μM in the presence of 5 nM PMA. Neither lysoPtdCho nor linoleic acid alone was capable of inducing the phagocytic activity. A similar result was obtained with snake venom phospholipase $A_2$ instead of lysoPtdCho and linoleic acid (Fig. 3*C*).

**Specificity of Lysophospholipids and Fatty Acids.** The stimulatory action of lysoPtdCho described above was specific. Other lysophospholipids, including lysophosphatidylinositol, lysophosphatidylserine, and lysophosphatidic acid, were inactive, except for lysophosphatidylethanolamine, which was slightly active to induce cell differentiation. Among various fatty acids, for instance, those with an 18-carbon chain, linolenic acid was most active, whereas linoleic and oleic acids were less active. On the other hand, stearic acid was inactive, indicating that unsaturated fatty acids were capable of intensifying the PMA-induced cell differentiation. Polyunsaturated fatty acids with much longer carbon chains, such as eicosapentaenoic and docosahexaenoic acids, were apparently toxic under comparable conditions, resulting in the cell lysis during the 24-hr incubation. The specificity of lysophospholipids and fatty acids for the induction of phagocytic activity is indicated in Table 1. Similar lipid specificities were observed when the expression of CD11b was determined as a marker of the cell differentiation.

**Morphological Change.** Treatment of HL-60 cells with sufficient amounts of PMA resulted in morphological changes—namely, cells became adhesive on a plastic dish and easily aggregated with one another, showing the shapes that are characteristic of macrophages. Under a submaximal amount of PMA (5 nM), which *per se* was insufficient to induce full differentiation of the cells within 24 hr, most cells were still round, although they were already adhesive on a plastic dish and, to some extent, aggregated with one another (Fig. 4*B*). Control cells are given in Fig. 4*A*. The addition of lysoPtdCho together with this amount of PMA induced the appearance of typical macrophages, indicating that lysoPtdCho promotes the differentiation of HL-60 cells (Fig. 4*C*). The addition of linoleic acid to the PMA-stimulated cells resulted in the almost complete differentiation on the morphological basis (Fig. 4*D*). Most cells adhered on the plastic dish, strongly aggregated with one another, showing the typical shapes characteristic of macrophages. Similarly, the cells were fully differentiated, when stimulated with a submaximal dose of PMA together with either lysoPtdCho plus linoleic acid (Fig. 4*E*) or the venom phospholipase $A_2$ (Fig. 4*F*). Neither lysoPtdCho, linoleic acid, nor phospholipase $A_2$ alone was active in this role, unless PMA was present.

## DISCUSSION

The enzyme termed phospholipase $A_2$, which hydrolyzes phospholipids (preferentially PtdCho) to produce fatty acids and 2-lysophospholipids, is ubiquitously present, and several soluble enzymes, intracellular and extracellular, have been identified in mammalian tissues (15). Some of these enzymes appear to cleave arachidonic acid selectively, but others liberate various fatty acids nonselectively, although the latter group of enzymes remains largely uncharacterized. Since receptor-mediated activation of phospholipase $A_2$ was recognized by Axelrod *et al.* (16), it is becoming clear that agonists that provoke inositol phospholipid hydrolysis usually also cause release of arachidonic acid (6, 7). The molecular species of fatty acids released upon receptor stimulation have not always been extensively studied, but it seems plausible that various cis-unsaturated fatty acids usually

Table 1. Specificity of lysophospholipids and fatty acids on HL-60 cell differentiation

| Addition | % phagocytic activity |
|---|---|
| Experiment 1 | |
| PMA | 27 |
| + lysoPtdCho | 40 |
| + lysoPtdEtn | 34 |
| + lysoPtdIns | 24 |
| + lysoPtdSer | 30 |
| + lysoPA | 29 |
| Experiment 2 | |
| PMA | 28 |
| + stearic acid | 29 |
| + oleic acid | 33 |
| + linoleic acid | 37 |
| + linolenic acid | 45 |

HL-60 cells ($2.0 \times 10^5$ cells per ml) were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum. The cells were incubated with various lysophospholipids or fatty acids (50 μM) in the presence of 5 nM PMA. After a 24-hr incubation, the phagocytic activity was determined. lysoPtdEtn, lysophosphatidylethanolamine; lysoPtdIns, lysophosphatidylinositol; lysoPtdSer, lysophosphatidylserine; lysoPA, lysophosphatidic acid.

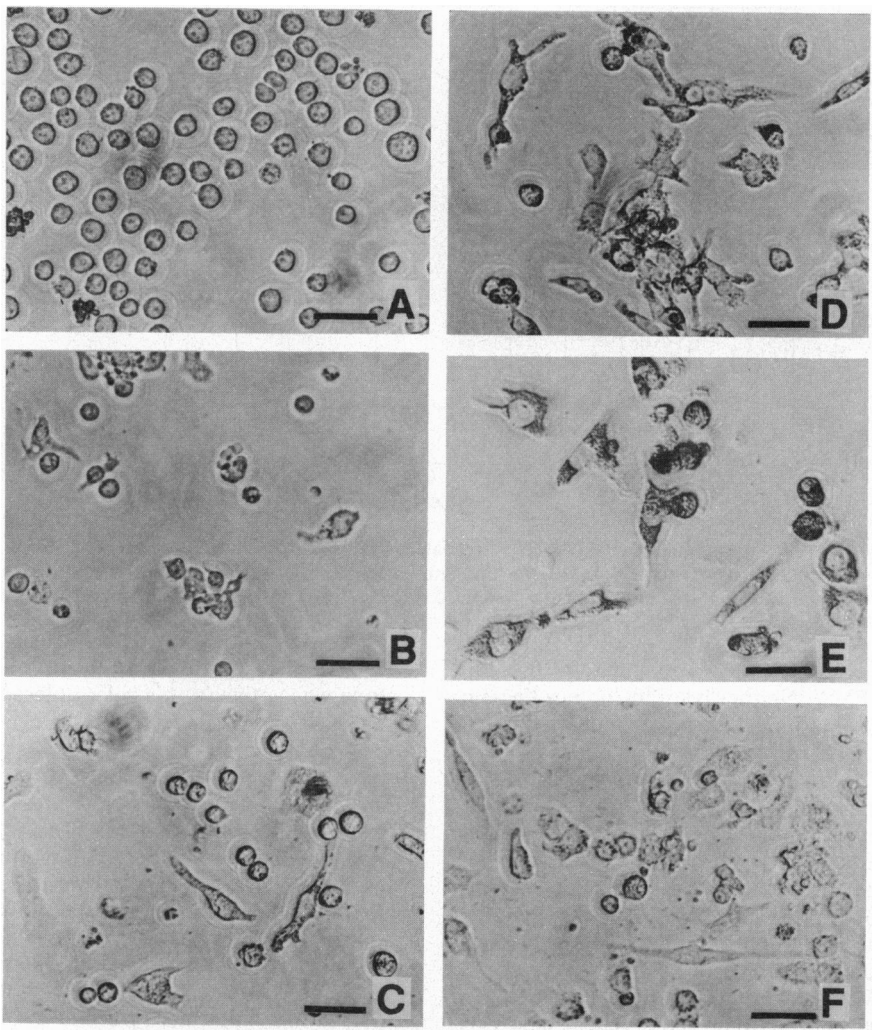


FIG. 4. Morphological changes of PMA-stimulated HL-60 cells incubated with lysoPtdCho, linoleic acid, or snake venom phospholipase $A_2$. HL-60 cells ($2.0 \times 10^5$ cells per ml) were incubated with either 50 $\mu$M lysoPtdCho (*C*), 50 $\mu$M linoleic acid (*D*), 50 $\mu$M lysoPtdCho plus 50 $\mu$M linoleic acid (*E*), or 50 units of *C. adamanteus* venom phospholipase $A_2$ per ml (*F*) in the presence of 5 nM PMA. As control experiments, the cells were incubated with 5 nM PMA alone (*B*) or without any additions (*A*). Cells were photographed after 24 hr. (Bars = 30 $\mu$m.)

esterified at the position 2 of membrane phospholipids are also liberated (17).

Preceding reports (12, 13) have shown that cis-unsaturated fatty acids greatly potentiate 1,2-$DiC_8$- or PMA-induced platelet activation, whereas lysoPtdCho, the other product of PtdCho hydrolysis, significantly enhances 1,2-$DiC_8$- or PMA-induced T-lymphocyte proliferation. In platelets lysoPtdCho is far less effective than fatty acids, and, inversely, in T-lymphocytes cis-unsaturated fatty acids are almost inert. The results obtained in the present studies show that in HL-60 cells cis-unsaturated fatty acids and lysoPtdCho are capable of potentiating the cellular response to 1,2-$DiC_8$ or PMA, resulting in the differentiation to macrophages. Relative efficiencies of lysoPtdCho and cis-unsaturated fatty acids for the potentiation of cellular responses may be partly due to different metabolic rates of these lipids within the various cell types employed.

Kinetic analysis with purified enzyme preparations indicates that cis-unsaturated fatty acids greatly enhance the activation of the $\alpha$, $\beta$, and $\gamma$ subspecies (cPKC) and allow these enzymes to exhibit nearly full activity at $<1$ $\mu$M $Ca^{2+}$ concentrations (18). DAG is absolutely needed for this activation. The $\delta$ subspecies (nPKC), which is expressed in many tissues and cell types, including HL-60 cells, is insensitive to fatty acids and $Ca^{2+}$ (19). These observations together with those described earlier (13) suggest that the potentiation of HL-60 cell differentiation by cis-unsaturated fatty acids may result from the enhancement of DAG-dependent activation of the $\alpha$ or $\beta$ subspecies, both of which are expressed in this cell line (20).

On the other hand, the mechanism of action of lysoPtdCho described above remains relatively unclear. Oishi *et al.* (21) have reported that lysoPtdCho activates PKC at higher concentrations of $Ca^{2+}$, whereas it inhibits the enzyme at lower concentrations of this cation. Recent analysis in this laboratory has revealed that lysoPtdCho at lower concentrations enhances DAG-dependent activation of cPKC but inversely inhibits nPKC activation (22). It is possible that lysoPtdCho may exert its biological effects, at least in part, through the enhancement of DAG-dependent activation of the $\alpha$ or $\beta$ subspecies. The $\gamma$ subspecies is not expressed in HL-60 cells (20).

Several lines of evidence obtained from studies with intact and permeabilized cell systems suggest that the intracellular phospholipases $A_2$ may be activated by many $Ca^{2+}$-mobilizing hormones, neurotransmitters, and some growth factors (for reviews, see refs. 6 and 7). An arachidonic acid-selective high molecular weight phospholipase $A_2$ has recently been shown to be activated by direct phosphorylation by PKC and microtubule-associated protein 2 kinase (23, 24), whereas the biochemical mechanism of activation of the

nonselective type of phospholipase $A_2$ remains to be clarified.

The biological activity of cis-unsaturated fatty acids to enhance cellular responses has recently been described for the murine T helper cell response to interleukin 1 (25) and for the presynaptic metabotropic receptor response to glutamate (26). The latter observation seems to be of particular significance, since it has been frequently proposed that cis-unsaturated fatty acids such as arachidonic and oleic acids play a role in the maintenance of long-term potentiation in the hippocampus (27, 28). Our previous studies have shown that secretory phospholipase $A_2$ (group II) potentiates T-lymphocyte activation, which is induced by PKC activation, and thereby participates in the propagation of inflammatory reactions (29). The extracellular phospholipase $A_2$ is also found in glial cells (30). It is attractive to surmise, then, that phospholipase $A_2$ takes part in the signal transduction through PKC in various cellular responses.

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1. Collins, S. J. (1987) *Blood* **70,** 1233–1244.
2. Kreutter, D., Caldwell, A. B. & Morin, M. J. (1985) *J. Biol. Chem.* **260,** 5979–5984.
3. Yamamoto, S., Gotoh, H., Aizu, E. & Kato, R. (1985) *J. Biol. Chem.* **260,** 14230–14234.
4. Morin, M. J., Kreutter, D., Rasmussen, H. & Sartorelli, A. C. (1987) *J. Biol. Chem.* **262,** 11758–11763.
5. Aihara, H., Asaoka, Y., Yoshida, K. & Nishizuka, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11062–11066.
6. Asaoka, Y., Nakamura, S., Yoshida, K. & Nishizuka, Y. (1992) *Trends Biochem. Sci.* **17,** 414–417.
7. Nishizuka, Y. (1992) *Science* **258,** 607–614.
8. Pelech, S. L. & Vance, D. E. (1989) *Trends Biochem. Sci.* **14,** 28–30.
9. Exton, J. H. (1990) *J. Biol. Chem.* **265,** 1–4.
10. Billah, M. M. & Anthes, J. C. (1990) *Biochem. J.* **269,** 281–291.
11. Liscovitch, M. (1992) *Trends Biochem. Sci.* **17,** 393–399.
12. Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y. & Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6447–6451.
13. Yoshida, K., Asaoka, Y. & Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6443–6446.
14. Blair, O. C., Carbone, R. & Sartorelli, A. C. (1986) *Cytometry* **7,** 171–177.
15. Dennis, E. A., Rhee, S. G., Billah, M. M. & Hannun, Y. A. (1991) *FASEB J.* **5,** 2068–2077.
16. Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988) *Trends Neurosci.* **11,** 117–123.
17. Conquer, J. & Mahadevappa, V. G. (1991) *J. Lipid Mediators* **3,** 113–123.
18. Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5149–5153.
19. Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y. & Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1592–1596.
20. Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, I., Mikawa, K. & Nishizuka, Y. (1990) *FEBS Lett.* **263,** 31–34.
21. Oishi, K., Raynor, R. L., Charp, P. A. & Kuo, J. F. (1988) *J. Biol. Chem.* **263,** 6865–6871.
22. Sasaki, Y., Asaoka, Y. & Nishizuka, Y. (1993) *FEBS Lett.* **320,** 47–51.
23. Lin, L.-L., Lin, A. Y. & Knopf, J. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6147–6151.
24. Nemenoff, R. A., Winitz, S., Qian, N.-X., Putten, V. V., Johnson, G. L. & Heasley, L. E. (1993) *J. Biol. Chem.* **268,** 1960–1964.
25. Bomalaski, J. S., Steiner, M. R., Simon, P. L. & Clark, M. A. (1992) *J. Immunol.* **148,** 155–160.
26. Herrero, I., Miras-Portugal, M. T. & Sánchez-Prieto, J. (1992) *Nature (London)* **360,** 163–166.
27. Linden, D. J., Sheu, F.-S., Murakami, K. & Routtenberg, A. (1987) *J. Neurosci.* **7,** 3783–3792.
28. Bliss, T. V. P. & Collingridge, G. L. (1993) *Nature (London)* **361,** 31–39.
29. Asaoka, Y., Yoshida, K., Sasaki, Y., Nishizuka, Y., Murakami, M., Kudo, I. & Inoue, K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 716–719.
30. Oka, S. & Arita, H. (1991) *J. Biol. Chem.* **266,** 9956–9960.