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Role of lysophosphatidylcholine in T-lymphocyte activation: Involvement of phospholipase A_2 in signal transduction through protein kinase C

(phosphatidylcholine/diacylglycerol/phospholipase C)

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ABSTRACT **2-Lysophosphatidylcholine (lysoPtdCho), a product of the hydrolysis of phosphatidylcholine catalyzed by phospholipase A_2, greatly potentiates the activation of human resting T lymphocytes that is induced by a membrane-permeant diacylglycerol plus a calcium ionophore, as determined by the expression of the α subunit of the interleukin 2 receptor and thymidine incorporation into DNA. LysoPtdCho *per se* is inactive unless both diacylglycerol and a calcium ionophore are present. This effect of lysoPtdCho is also observed when diacylglycerol is replaced by a tumor-promoting phorbol ester. Other lysophosphatides including lysophosphatidylserine, lysophosphatidylinositol, and lysophosphatidic acid are inert except for lysophosphatidylethanolamine, which is far less effective than lysoPtdCho. Tracer experiments with radioactive choline indicate that, when T lymphocytes are stimulated with an antigenic signal, lysoPtdCho is indeed produced in a time-dependent fashion, although the concentration of this lysophospholipid accumulated remains to be quantitated. It suggests that phospholipase A_2 is directly involved in the signal transduction pathway through protein kinase C to induce long-term cellular responses.**

A single dose of a tumor-promoting phorbol ester, together with a calcium ionophore, can induce T-lymphocyte activation, as determined by secretion of interleukin 2, expression of the α subunit of the interleukin 2 receptor (IL-2Rα), and incorporation of thymidine into DNA (for a review, see ref. 1). On the other hand, a single dose of a membrane-permeant diacylglycerol (DAG) is normally insufficient to induce cell activation due to its rapid metabolism within the cell, and it is known that sustained activation of protein kinase C (PKC) by a large dose or repeated doses of a membrane-permeant DAG is essential to induce activation of T lymphocytes (2, 3). The formation of DAG from receptor-mediated hydrolysis of inositol phospholipids is, however, normally transient, and recent evidence strongly suggests that, at relatively later phases in cellular responses, DAG is produced from signal-induced breakdown of phosphatidylcholine (PtdCho), which is initiated presumably by the activation of phospholipase D (for reviews, see refs. 4 and 5). Previous reports from this laboratory (6, 7) suggest that cis-unsaturated fatty acids greatly enhance PKC activation when DAG is available. It has also been briefly described that 2-lysophosphatidylcholine (lysoPtdCho), the other product of receptor-mediated hydrolysis of PtdCho by phospholipase A_2, synergizes with a membrane-permeant DAG to activate human resting T lymphocytes (8). In fact, activation of both phospholipase C and phospholipase A_2 by a single agonist has been reported by Axelrod and coworkers (9). The receptor-mediated degradation of various membrane phospholipids, therefore, may be important in causing cellular responses such as cell proliferation and differentiation. The studies presented herein were undertaken to explore further the detailed kinects of the lysoPtdCho action on T-lymphocyte activation.

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MATERIALS AND METHODS

Materials. Human peripheral resting T lymphocytes were prepared from venous blood donated by healthy volunteers. Accessory cells were removed as much as possible as described (3). The medium used for T-lymphocyte culture was RPMI 1640 supplemented with 5% (vol/vol) autologous serum. Radioactive 1,2-dioctanoylglycerol (DiC_8) (racemic 1,2-di[1-^{14}C]octanoylglycerol; 27 mCi/mmol; 1 Ci = 37 GBq), lysoPtdCho (L-[1-^{14}C]palmitoyllysoPtdCho; 56 mCi/mmol), 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 DuPont.

1,2-DiC_8 was a product of Nakalai Tesque (Kyoto). This preparation consists of 95% DL-1,2-DiC_8 and 5% 1,3-DiC_8 as estimated by TLC. L-α-DecanoyllysoPtdCho, L-α-myristoyllysoPtdCho, L-α-palmitoyllysoPtdCho, L-α-stearoyllysoPtdCho, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, and L-α-oleoyllysophosphatidic acid were obtained from Sigma. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from LC Services (Woburn, MA) and Calbiochem–Behring, respectively. Other chemicals were obtained from commercial sources.

Determination of IL-2Rα Expression. Cells seeded at a density of 5.0×10^5 cells per ml in RPMI 1640 containing 5% autologous serum were stimulated as specified in each experiment. After incubation for 16 hr, the cells were washed, resuspended in phosphate-buffered saline (PBS) at a density of 1.0×10^7 cells per ml, and treated for 30 min at 4°C with a fluorescein-conjugated anti-CD25 monoclonal antibody that recognizes IL-2Rα (Beckton Dickinson) at an appropriate dilution. The cells were washed again and resuspended in PBS. The fluorescence intensity was determined by analysis of 5000 cells with a flow cytometer (Cyto ACE-150; Japan Spectroscopic, Tokyo).

Thymidine Incorporation Assay. Cells (5×10^5 cells in 1 ml of culture medium per well) were stimulated as specified in each experiment and incubated for 30 hr. For the last 6 hr of the incubation, the cells were exposed to [^{3}H]thymidine (0.5 μCi per well). The acid-precipitable material was collected on a glass filter (Whatman; GF/A), and the radioactivity was

Abbreviations: IL-2Rα, α subunit of the interleukin 2 receptor; DAG, diacylglycerol; PKC, protein kinase C; PtdCho, phosphatidylcholine; lysoPtdCho, 2-lysophosphatidylcholine; DiC_8, dioctanoylglycerol; PMA, phorbol 12-myristate 13-acetate.

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quantitated with a liquid scintillation spectrometer as described (8).

Metabolism of 1,2-DiC$_8$ and LysoPtdCho. Metabolism of 1,2-DiC$_8$ was analyzed with DL-1,2-[^{14}C]DiC$_8$ as described (3). The radioactive 1,2-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 $\times$ 10^5 cpm per ml). After incubation, lipids were extracted directly from the cell suspension by the method of Bligh and Dyer (10) with 1 M HCl as an upper phase. Chloroform-soluble material was separated by TLC on a Merck silica gel 60 plate with chloroform/acetone, 96:4 (vol/vol) as the 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).

The radioactive lysoPtdCho was dispersed in PBS and added to the cell suspension at a final concentration of 50 μM (2–3 $\times$ 10^5 cpm/ml). After incubation, lipids were extracted as described above and separated by TLC on a Merck silica gel 60 plate with chloroform/methanol/water, 65:25:4 (vol/vol) as the solvent system. Nonradioactive palmitoyllysoPtdCho, palmitic acid, and PtdCho were run as authentic markers. Each spot was visualized by I_2 vapor, and the radioactivity of the spots corresponding to lysoPtdCho and its metabolites was quantitated as described above.

Measurement of the Intracellular Calcium Ion Concentration. The intracellular Ca^{2+} concentration was measured as described by Poenie *et al.* (11) with a slight modification. To a suspension of resting T lymphocytes (5 $\times$ 10^5 cells per ml) in 10 mM Hepes at pH 7.4 containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM glucose (HBS buffer), a solution of the acetoxymethyl ester of fura-2 in 1 mM dimethyl sulfoxide was added to give a final concentration of 5 μM of this compound. 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 $\times$ 10^5 cells per ml. The extracellular Ca^{2+} concentration was adjusted to a level equivalent to that in RPMI 1640 containing 5% autologous serum. The cells were stimulated as specified. The fluorescence intensities at 350 nm and 385 nm were measured by using a CAF-100 calcium analyzer (Japan Spectroscopic, Tokyo).

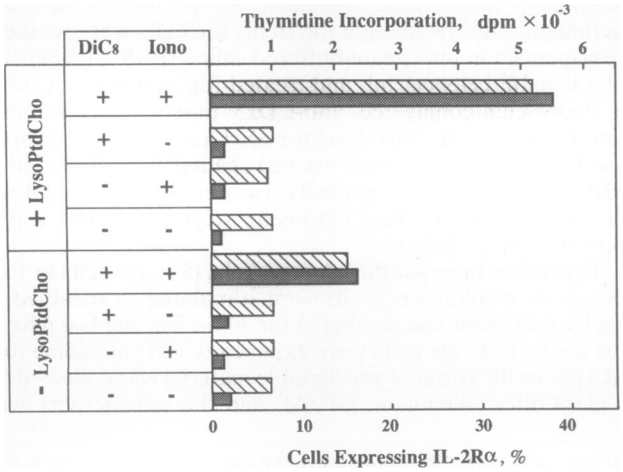

FIG. 1. Effect of lysoPtdCho on T-lymphocyte activation induced by 1,2-DiC$_8$ and ionomycin. Resting T lymphocytes (5 $\times$ 10^5 cells per ml) were stimulated with 1,2-DiC$_8$ (50 μM) plus ionomycin (0.5 μM) and lysoPtdCho (50 μM), as indicated. After incubation, the IL-2Rα expression (shaded bars) and [^{3}H]thymidine incorporation (hatched bars) were determined. Iono, ionomycin.

LysoPtdCho Formation in T Lymphocytes. T lymphocytes (1 $\times$ 10^7 cells per ml) were labeled with [^{3}H]choline (3 μCi/ml) for 24 hr as described by Slivka and Insel (12). After incubation, the cells were washed three times and suspended in PBS at a density of 1.0 $\times$ 10^7 cells per ml. The cells were then stimulated with an anti-CD3 monoclonal antibody (10 μg/ml) (Immunotech, Marseille, France). At the times indicated, lipids were extracted and separated as described above. Nonradioactive lysoPtdCho was run simultaneously as an authentic marker. Lipids were visualized by I_2 vapor, and the spot corresponding to lysoPtdCho was scraped off. The radioactivity was measured by a liquid scintillation spectrometer.

RESULTS

LysoPtdCho and T-Lymphocyte Activation. Sustained PKC activity appears to be essential for T-lymphocyte activation as described (2, 3). The extent of this cell activation by a single dose of 1,2-DiC$_8$ is, therefore, inversely related to the cell density and directly proportional to the amount of 1,2-DiC$_8$ added to the incubation medium, because this DAG is taken up by the cell and metabolized very quickly (3). It was found in the present studies that the addition of lysoPtdCho to the incubation medium greatly potentiated the cell activation that was induced by 1,2-DiC$_8$ plus ionomycin, as measured by IL-2Rα expression and thymidine incorporation into DNA (Fig. 1). LysoPtdCho *per se* was totally inactive unless both 1,2-DiC$_8$ and ionomycin were present. It is important to note that, when the cells were stimulated by PMA instead of 1,2-DiC$_8$, a similar potentiation of cell activation by lysoPtdCho was observed.

LysoPtdCho Concentration and Specificity. The precise concentration of lysoPtdCho that was actually effective for this potentiation was difficult to be measured, since the incubation medium contained serum, which bound and removed most of lysophospholipid available for this cell activation. However, the stimulatory effect was evident at 10 μM lysoPtdCho and appeared to be maximum at 100 μM when

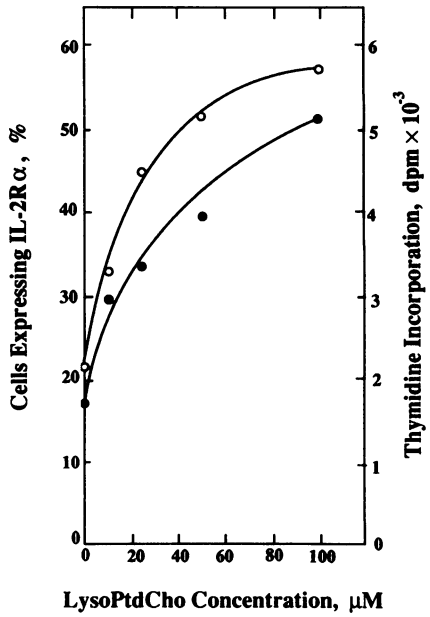

FIG. 2. Effect of lysoPtdCho concentration on T-lymphocyte activation by 1,2-DiC$_8$ and ionomycin. Resting T lymphocytes (5 $\times$ 10^5 cells per ml) were stimulated with 1,2-DiC$_8$ (50 μM) plus ionomycin (0.5 μM) in the presence of various concentrations of lysoPtdCho as indicated. IL-2Rα expression (●) and thymidine incorporation (○) were determined.

the medium contained 5% autologous serum (Fig. 2). Perhaps, lysoPtdCho was effective at <10 μM, because even at this concentration a large part of this lipid remained in the cell culture medium in association with serum protein. At concentrations >100 μM, lysoPtdCho was toxic and caused cell lysis.

The stimulatory action described above was specific for lysoPtdCho. All other lysophospholipids thus far tested were inactive under comparable conditions except for lysophosphatidylethanolamine which was 10–20% as active as lysoPtdCho (Fig. 3). Among various molecular species of lysoPtdCho, myristoyllysoPtdCho, palmitoyllysoPtdCho, and stearoyllysoPtdCho were all active, whereas decanyollysoPtdCho was far less effective in T-lymphocyte activation.

1,2-DiC_8 and Ionomycin Concentration. The activation of T lymphocytes absolutely required both 1,2-DiC_8 and ionomycin, irrespective of the presence or absence of lysoPtdCho. LysoPtdCho did not significantly affect the apparent half-maximum concentration of 1,2-DiC_8 that was needed for cell activation (Fig. 4*A*). Measurement of the intracellular Ca^{2+} concentration with the fura-2 procedure confirmed that lysoPtdCho did not increase the sensitivity of this T-lymphocyte response to Ca^{2+} and did not elevate the intracellular Ca^{2+} concentration (Fig. 4*B*).

Metabolism of LysoPtdCho. 1,2-DiC_8 added to a suspension of T lymphocytes disappeared very quickly, with the concomitant accumulation of its metabolic products. This disappearance of 1,2-DiC_8 appeared to be due to its hydrolysis to produce mostly octanoic acid (Fig. 5). The rate of hydrolysis was not affected by the coexistence of lysoPtdCho in the incubation medium.

LysoPtdCho, 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 protein even after 6 hr. Similar results were obtained both in the presence and the absence of 1,2-DiC_8 and ionomycin.

Another set of experiments showed that lysoPtdCho was no longer effective after a prolonged period of time when 1,2-DiC_8 completely disappeared from the incubation medium (Fig. 6*A*). In contrast, when 1,2-DiC_8 was replaced by PMA, lysoPtdCho was always able to enhance the cell activation, presumably because PMA was metabolically stable and remained in the incubation medium (Fig. 6*B*).

LysoPtdCho Formation by Physiological Signals. It has been well documented that antigenic signals cause inositol phospholipid hydrolysis to produce DAG (1). A preliminary

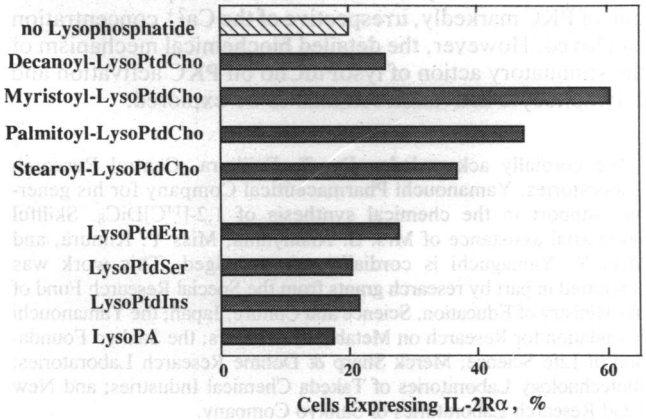

FIG. 3. Specificity of various lysophospholipids on T-lymphocyte activation. T lymphocytes were stimulated with various lysophospholipids (50 μM) in the presence of 1,2-DiC_8 (50 μM) plus ionomycin (0.5 μM), as indicated. After incubation, the expression of IL-2Rα was measured as described. LysoPtdEtn, lysophosphatidylethanolamine; LysoPtdSer, lysophosphatidylserine; LysoPtdIns, lysophosphatidylinositol; LysoPA, L-α-oleoyllysophosphatidic acid.

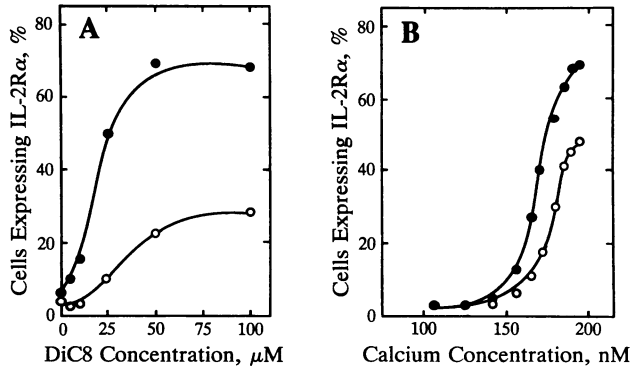

FIG. 4. IL-2Rα expression as a function of 1,2-DiC_8 or intracellular Ca^{2+} concentration in the presence and absence of lysoPtdCho. The experimental conditions were essentially identical with those given in Fig. 1, except that various amounts of 1,2-DiC_8 were added (*A*) or various amounts of ionomycin were added to give intracellular Ca^{2+} concentrations between 106 nM (the resting conditions) and 196 nM as indicated (*B*). The intracellular Ca^{2+} concentration after the addition of various amounts of ionomycin was measured as described and plotted versus IL-2Rα expression. ●, in the presence of lysoPtdCho (50 μM); ○, in the absence of lysoPtdCho.

experiment with radioactive choline as a tracer showed that, after the T lymphocytes were stimulated with an anti-CD3 antibody, lysoPtdCho gradually increased significantly (Fig. 7). It was not possible in this experiment to determine the concentration of lysoPtdCho that accumulated.

DISCUSSION

Inositol phospholipid hydrolysis, initiated by either receptor stimulation or Ca^{2+}-gate opening, was previously thought to be the sole mechanism by which DAG is produced for linking extracellular signals to intracellular events through PKC activation (13). It has recently become evident, however, that phospholipase D may also take part in producing DAG from PtdCho in relatively later phases of cellular responses, par-

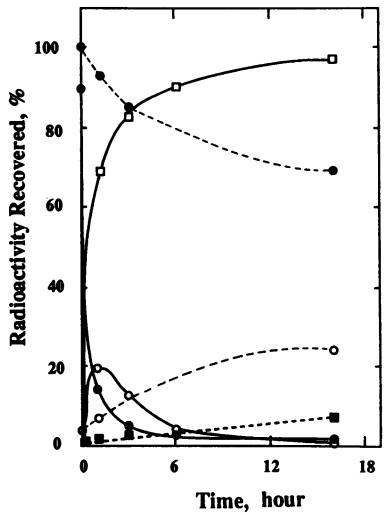

FIG. 5. Metabolism of 1,2-DiC_8 and lysoPtdCho in T lymphocytes. 1,2-[^{14}C]DiC_8 (50 μM; 2–3 × 10^5 cpm/ml) or [^{14}C]lysoPtdCho (50 μM; 2–3 × 10^5 cpm/ml) was added to the cell suspension (5 × 10^5 cells per ml) in RPMI 1640 supplemented with 5% autologous serum. At the various times indicated, lipids 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. ●—●, 1,2-DiC_8; ○—○, 1,3-DiC_8; □—□, total metabolites of 1,2-DiC_8. ●- - -●, lysoPtdCho; ○- - -○, palmitic acid; ■- - -■, PtdCho.

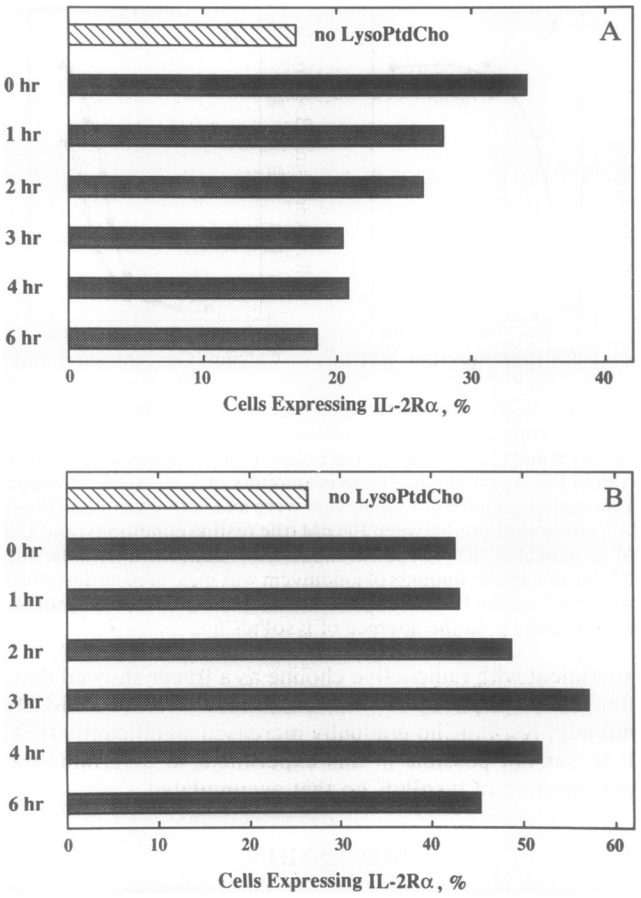

FIG. 6. Effect of delayed addition of lysoPtdCho on IL-2Rα expression induced by 1,2-DiC_8 or PMA in the presence of ionomycin. T lymphocytes were stimulated with 50 μM 1,2-DiC_8 (*A*) or 1 nM PMA (*B*) in the presence of ionomycin (0.5 μM in *A*; 0.3 μM in *B*). LysoPtdCho (50 μM) was added at the time indicated after the addition of ionomycin plus either 1,2-DiC_8 or PMA. After incubation for a total of 16 hr, the expression of IL-2Rα was quantitated. No lysoPtdCho represents the IL-2Rα expression induced by ionomycin plus either 1,2-DiC_8 (*A*) or PMA (*B*) without lysoPtdCho.

ticularly those involving long-acting signals such as some growth factors (for reviews, see refs. 4 and 5). The results presented in this paper, together with those described earlier (6–8), indicate that signal-induced activation of phospholipase A_2 generates two additional biologically active molecules from PtdCho, cis-unsaturated fatty acid and lysoPtdCho, both of which greatly enhance subsequent cellular responses. It may be emphasized that the actions of these two molecules absolutely require the presence of DAG, indicating that PKC plays a central role in the cell surface signal transduction for cellular regulation. However, the activity of cis-unsaturated fatty acids and lysoPtdCho for the potentiation of cellular responses greatly varies with the cell type examined. For activation of platelets, lysoPtdCho is far less effective than cis-unsaturated fatty acids, whereas for the activation of T lymphocytes, lysoPtdCho is more effective than cis-unsaturated fatty acids. The reason for such differences is not clear presently, but multiple factors may be considered. For instance, the precise concentration of lysoPtdCho actually effective in enhancing the T-lymphocyte activation is difficult to be measured, because the incubation medium contains serum, which may trap this lysophospholipid and prevent the uptake of this lipid by the cell. However, it remains to be determined the exact concentration of lysoPtdCho endogenously produced after cell stimulation by physiological signals.

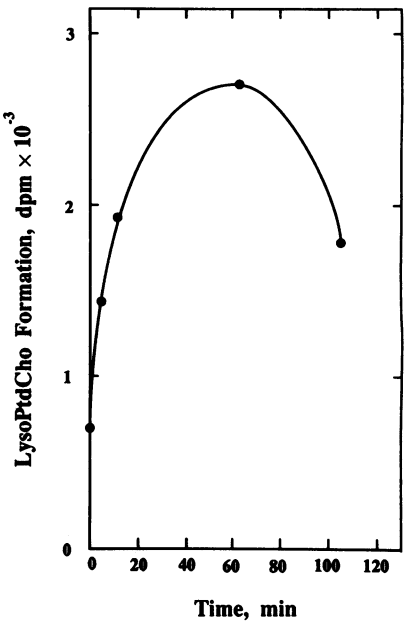

FIG. 7. Formation of lysoPtdCho in T lymphocytes stimulated with an antigenic signal. T lymphocytes (1×10^7 cells per ml) were labeled with [^{3}H]choline (3 μCi/ml) for 24 hr. After washing, the cells (1×10^7 cells per ml) were stimulated with an anti-CD3 monoclonal antibody (10 μg/ml). At the various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

LysoPtdCho does not appear to act simply as a detergent or calcium ionophore in the experiments presented above, although the possibility that it facilitates the penetration of 1,2-DiC_8 into the cell membrane cannot be ruled out. Marquardt and Walker (14) have very recently found that lysoPtdCho has the ability to potentiate antigen-stimulated secretion of mouse mast cells and also to induce by itself the translocation of PKC from the cytosol to the membrane. The relation of their observations to the results presented in this paper is not clear at present.

An early report by Kuo and coworkers (15) indicated that lysoPtdCho exerts a biphasic action on the enzymatic activity of PKC, inhibitory or stimulatory, depending upon the Ca^{2+} concentration in the reaction mixture. Although these observations are fully reproducible, lysoPtdCho at much lower concentrations always enhances the DAG-dependent activation of PKC markedly, irrespective of the Ca^{2+} concentration employed. However, the detailed biochemical mechanism of the stimulatory action of lysoPtdCho on PKC activation and T-lymphocyte activation remains to be explored.

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