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Lysophosphatidylcholine Metabolism to 1,2-Diacylglycerol in Lymphoblasts: Involvement of a Phosphatidylcholine-Hydrolyzing Phospholipase C†

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ABSTRACT: We have previously described the chemoattraction of lymphoblasts by lysophosphatidylcholine [Hoffman, R. D., et al. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3285-3289]. In studying the mechanism of chemoattraction it was found that lysophosphatidylcholine was metabolized to 1,2-diacylglycerol by the lymphoblastic cell line 6C3HED. One route of metabolism involves the acylation of lysophosphatidylcholine to phosphatidylcholine with subsequent hydrolysis to 1,2-diacylglycerol and phosphocholine by the action of phospholipase C. The increase in cellular 1,2-diacylglycerol was established by metabolic experiments using [^{14}C]glycerol-labeled lysophosphatidylcholine and by mass measurements of 1,2-diacylglycerol. The presence of a phosphatidylcholine-hydrolyzing phospholipase C was confirmed in 6C3HED cell homogenates. In intact cells, lysophosphatidylcholine induced a pattern of protein phosphorylation similar to those of 1,2-dioctanoylglycerol and phorbol 12-myristate 13-acetate, two known activators of protein kinase C. This pathway of lysophosphatidylcholine metabolism, which involves a phosphatidylcholine-hydrolyzing phospholipase C, may be important in the activation of protein kinase C independent of inositol phospholipid hydrolysis.

The activation of protein kinase C, a phospholipid- and calcium-dependent kinase, is believed to play an important role in receptor-mediated signal transduction [for review see Nishizuka (1984, 1986)]. In a variety of cells, activation of protein kinase C leads to numerous biological responses including secretion, ion fluxes, superoxide generation, proliferation, and differentiation (Nishizuka, 1986; Ashendel, 1985). One of the factors modulating the activation of protein kinase C is 1,2-diacylglycerol (DAG),[1] which is believed to operate by reducing the enzyme's requirement for calcium to a physiologic range (Kikkawa et al., 1982). In many cells DAG has been shown to be generated from inositol phospholipids

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[1] Abbreviations: DAG, diacylglycerol; LPC, lysophosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TLC, thin-layer chromatography; [^{14}C]FA-LPC, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; [^{14}C]choline-LPC, 1-palmitoyl-*sn*-glycero-3-phospho[N-*methyl*-^{14}C]choline; [^{14}C]glycerol-LPC, 1-palmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; PFB, phosphate-free buffer.

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by the action of phosphatidylinositol (PI) specific phospholipase C in response to receptor stimulation (Nishizuka, 1983; Majerus et al., 1985; Cockroft, 1987).

A number of recent studies have shown that DAG may also be derived from sources other than phosphatidylinositol. Besterman et al. (1986) reported that production of DAG and phosphocholine was increased in 3T3-L1 and HL60 cells following stimulation with phorbol esters, suggesting the hydrolysis of phosphatidylcholine by phospholipase C. A similar response to phorbol esters has been described in canine kidney cells (Daniel et al., 1986) and Swiss 3T3 fibroblasts (Muir & Murray, 1987). Irving and Exton (1987) have also reported the stimulation of phosphocholine release from rat liver membranes by guanine nucleotides and P_2-purinergic agonists. These studies indicate that DAG is produced from sources other than PI during cell activation and further suggest the involvement of a phosphatidylcholine-hydrolyzing enzyme.

We have previously described the chemoattraction of lymphoblastic cells by lysophosphatidylcholine (LPC) (Hoffman et al., 1982). In our studies of the mechanism by which LPC stimulates cell motility, we examined potential metabolites of LPC and found cell-permeable analogues of DAG to be active (Wright et al., 1988a). Whereas the acylation of LPC to phosphatidylcholine has been well described in a variety of cells (Lands, 1960; Brumley & Van den Bosch, 1977; Chaudhary, 1982; Metz, 1987; Gross et al., 1983; Tamura et al., 1985; Chilton et al., 1987; Kramer et al., 1984), the existence of phosphatidylcholine-hydrolyzing phospholipase C activity in mammalian cells has only recently been appreciated (Matsuzawa & Hostetler, 1980; Wolf & Gross, 1985; Sheikhnejad & Srivastava, 1986). We have examined whether LPC can be metabolized to DAG in lymphoblastic cells and whether this process leads to the activation of protein kinase C. Our findings indicate that LPC is acylated to phosphatidylcholine and that phosphatidylcholine so formed is hydrolyzed to DAG. The formation of DAG from LPC was established in experiments using radiolabeled LPC as well as measurements of DAG mass levels. In addition, we observed that LPC induces a pattern of protein phosphorylation similar to those of phorbol 12-myristate 13-acetate (PMA) and DAG, two activators of protein kinase C (Nishizuka, 1984, 1986). We believe that this pathway may represent an important mechanism by which DAG is generated from sources other than inositol phospholipids.

Experimental Procedures

Materials. 1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphocholine (61 mCi/mmol), [1-^{14}C]palmitic acid (58 mCi/mmol), L-[U-^{14}C]glycerol 3-phosphate (170 mCi/mmol), [^{14}C]methyl iodide (58 mCi/mmol), and [^{32}P]orthophosphate (carrier-free, 8 mCi/mL) were purchased from Amersham Corp. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from New England Nuclear. Anasil O TLC plates were obtained from Foxboro. Silica gel HL plates and tapered silica gel G preparative plates were supplied by Analtech. Organic solvents were purchased from Burdick-Jackson Laboratories or Aldrich and were used without further purification. Lipids were obtained from Serdary Research Laboratories and Avanti Polar Lipids. Unless specified otherwise, reagents were purchased from Aldrich or Sigma and were of the highest available purity.

Cell Culture. The origin and maintenance of the cultured mouse thymic lymphoma 6C3HED has been described previously (Pasternack et al., 1978). Cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Analysis of Lysophosphatidylcholine (LPC) and Fatty Acid Metabolism in 6C3HED Cells. 6C3HED cells were suspended at 5×10^6/mL in minimal essential medium (MEM) with Earle's salts containing 0.5% (w/v) bovine serum albumin (Cohn fraction V, Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 70 mM Na-HEPES, pH 7.5 (serum-free medium). For each sample, labeled LPC (approximately 1 μCi, 50 nmol) or labeled palmitic acid (approximately 1 μCi, 50 nmol) was dried under a stream of nitrogen and suspended in 500 μL of serum-free medium. The reaction was initiated by mixing equal volumes of cell suspension and medium containing radiolabel to make a final volume of 1 mL, followed by incubation at 37 °C in a shaking water bath. The final concentration of labeled LPC or fatty acid was 50 μM. Bovine serum albumin was present at 61.3 μM. At various time points, cells were pelleted by centrifugation (1000*g* for 2 min), and lipid and water-soluble fractions were extracted by the method of Bligh and Dyer (1959). In [^{14}C]palmitic acid labeled LPC and [^{14}C]palmitic acid experiments, the samples were extracted without separating cells from medium. In experiments using [^{14}C]-choline-labeled and [^{14}C]glycerol-labeled LPC, cell pellets were separated from media by centrifugation prior to extraction. This was necessary for analysis of the cellular water-soluble products from these labeled compounds, because the salts in the medium interfered with TLC separation. The total recovered radioactivity included counts in the medium and cellular fractions. For phospholipid analysis, samples were applied to Anasil O TLC plates (Foxboro) and developed in chloroform/methanol/28% ammonium hydroxide/water (120:80:10:4, v/v). Alternatively, samples were analyzed by 2-dimensional TLC by applying them to Anasil O plates and developed in the first dimension with chloroform/methanol/28% ammonium hydroxide/water (as above) and chloroform/acetone/methanol/acetic acid/water (100:40:30:20:10, v/v) in the second dimension. Neutral lipids were separated by 1-dimensional TLC with isopropyl ether/acetic acid (96:4, v/v). Water-soluble fractions from cell pellets were dried under reduced pressure, dissolved in 50 μL of 50% ethanol, and applied to a silica gel HL plate (Analtech). The plate was developed with 0.5% NaCl/methanol/28% ammonium hydroxide (100:100:2, v/v). After removal of the solvents by evaporation, autoradiography was performed. The identity of the radioactive spots was confirmed by comparison with known standards visualized by iodine staining. The TLC plates were scraped into vials, and the distribution of radiolabel was quantitated by liquid scintillation counting.

Preparation of Choline-Labeled LPC. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[N-*methyl*-^{14}C]choline was prepared by methylation of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*-dimethylethanolamine with [^{14}C]methyl iodide in the presence of cyclohexylamine according to the method described by Stoffel (1975). This choline-labeled phosphatidylcholine was hydrolyzed to the lysophosphatide by *Naja naja* phospholipase A_2 according to established procedures (Kates, 1982).

Synthesis of Glycerol-Labeled LPC. L-[U-^{14}C]Glycerol 3-phosphate (0.4 mCi, 2.3 μmol, Amersham) was diluted with unlabeled glycerol 3-phosphate to a specific activity of 20 mCi/mmol and converted to the pyridinium salt by passage through a column of Dowex AG 501-X8–pyridinium. The sample was dried under reduced pressure and was rendered anhydrous by repeated addition and evaporation of dry pyridine. The glycerol 3-phosphate–pyridinium was redissolved in dry pyridine and was added to 0.15 mmol of palmitic anhydride and 0.09 mmol of (dimethylamino)pyridine according

to the method of Kingsley and Feigenson (1979). After 4 h at room temperature, water (0.2 mL) was added to decompose unreacted anhydrides. Solvents were removed under vacuum, and the crude product was dissolved in chloroform and applied to a column of silicic acid (Unisil). Free fatty acid was eluted with chloroform, and the labeled phosphatidic acid was eluted with chloroform/methanol (1:1, v/v). Yield was 81% on the basis of recovered radioactivity. The products were analyzed by TLC. The 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycerol 3-phosphate, now in the (dimethylamino)pyridinium form, was dried by repeated evaporation of dry pyridine and dissolved in 0.5 mL of dry pyridine. To this solution were added 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) and choline tetraphenylborate according to the method of Harbinson and Griffin (1984). The reaction vessel was flushed with nitrogen and stirred for 4 h at 30–35 °C. Following the reaction, water was added to decompose excess TPS, and the solvents were removed by evaporation at reduced pressure. The crude reaction mixture was dissolved in methylene chloride/methanol (1:1, v/v) and passed through a column of AG 501-X8 mixed bed ion exchange resin (Bio-Rad). The fraction containing labeled phosphatidylcholine was dried and further purified by silicic acid column chromatography and preparative TLC. Purity of the product was confirmed by 2-dimensional TLC as described above. 1,2-Dipalmitoyl-*sn*-[U-^{14}C]-glycero-3-phosphocholine was cleaved by snake venom phospholipase A_2, and the labeled LPC was purified as described above.

Measurement of Phospholipase C Activity. 6C3HED cells were washed twice in Hanks' balanced salt solution and resuspended at 5×10^7/mL in ice-cold buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 12 mM CHAPS, and 50 mM NaMOPS, pH 7.2. The cells were sonicated by using a Branson sonifier Model 250 (40 W for 30 s), and the resulting homogenate was maintained on ice until assayed. The typical reaction mixture for phospholipase C activity consisted of cell homogenate (100 μL) and substrate, 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine (0.08 μCi, 4 nmol), which had been dried from solution in chloroform and sonicated in 100 μL of buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 5 mM CHAPS, and 50 mM NaMOPS, pH 7.2. The substrate was warmed to 37 °C, and the reaction was started by the addition of crude enzyme (homogenate), vigorous mixing for 10 s, and incubation at 37 °C. The reaction was terminated by adding chilled chloroform/methanol (1:2, v/v), and lipids were extracted and separated as described above. The protein content of cell homogenates was determined by the BCA protein assay (Pierce). By this method there is approximately 1 mg of protein/10^7 cells. The final specific activity of substrate was based on added (4 nmol) and endogenous (approximately 50 nmol) phosphatidylcholine. The amount of endogenous phosphatidylcholine was determined by extracting the total lipids from aliquots of the cell homogenate followed by TLC separation (see above). The phosphatidylcholine fraction was quantitated by measurement of lipid phosphorus (Ames & Dubin, 1960).

Mass Measurement of DAG. 6C3HED cells were washed and resuspended in serum-free medium at a concentration of 4×10^6/mL. The cells were aliquoted into tubes and allowed to equilibrate for 30 min at 37 °C. 1-Palmitoyl-LPC (50 μM, final), palmitic acid (2.5 μM, final), or serum-free medium alone (warmed to 37 °C) was added to the cells followed by gentle mixing and incubation at 37 °C. The incubation was terminated by the addition of ice-cold chloroform/methanol (1:2, v/v). Lipid extraction and the mass measurement of DAG levels using diacylglycerol kinase (Lipidex) were performed as described previously (Preiss et al., 1986; Wright et al., 1988a).

Protein Phosphorylation. 6C3HED were labeled with [^{32}P]orthophosphate, and the phosphorylated proteins were analyzed essentially as described by Chaplin et al. (1980) except that cells were labeled in [^{32}P]orthophosphate for 10 min followed by an additional 10 min of incubation in the presence of a stimulant, e.g., PMA (10 nM), DAG (25 μM), or LPC (50 μM). Experiments with PMA indicated that longer prelabeling in [^{32}P]orthophosphate led to an increase in generalized protein phosphorylation and gradually obscured the specific effect of PMA. Briefly, 6C3HED cells were washed twice in phosphate-free buffer (PFB) containing 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM Mg_2SO_4, 5.5 mM D-glucose, 5 mg/mL bovine serum albumin (Cohn fraction V, Sigma), and 20 mM NaHEPES, pH 7.5. The cells were suspended at 5×10^6/mL in PFB and incubated at 37 °C for 15 min. After centrifugation (500*g* for 10 min), the cells were resuspended at 2.5×10^7/mL in PFB containing 0.25 mCi/mL [^{32}P]orthophosphate (carrier free), and 0.5-mL aliquots were placed in 1.5-mL microfuge tubes. The cells were labeled for 10 min at 37 °C, followed by the addition of stimuli (in PFB) or PFB in a volume of 0.5 mL. The tubes were mixed and incubated in a shaking water bath for 10 min, after which time they were cooled rapidly in an ice bath and centrifuged (15000*g* for 10 s, 4 °C). All subsequent steps were performed at 0–4 °C. The supernatants were aspirated, and 0.25 mL of lysis buffer (1% NP-40, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM EDTA, 2 mM PMSF, and 50 mM Tris-HCl, pH 7.5) was added to each tube followed by vigorous vortexing for 15 s and incubation on ice for 5 min. The tubes were then centrifuged (15000*g* for 15 min), and 0.1-mL aliquots of the supernatants (NP-40 soluble extracts) were removed and added to equal volumes of sample buffer (Laemmli, 1970). After boiling for 1 min, the samples were analyzed by electrophoresis in 7.5% SDS–polyacrylamide slab gels (Laemmli, 1970). The gels were stained and dried followed by autoradiography. Densitometric analysis of the autoradiographs was performed by using an LKB Ultrascan XL, scanning at 633 nm. Statistical analysis was performed by using Student's *t* test.

Because we modified the method of Chaplin et al. (1980) by shortening the preincubation of 6C3HED cells in [^{32}P]-orthophosphate to 10 min, we checked whether the specific activity of ATP reached steady state during the 10-min prelabeling period and whether LPC had any effect on this steady state. At various times during a 30-min incubation of 6C3HED cells in [^{32}P]orthophosphate, nucleotides were extracted (De Abreu et al., 1982) and fractionated (Torrance & Whittaker, 1979), and the specific activity of ATP was determined. In three separate experiments, the specific activity of ATP increased rapidly during the first 8 min, reaching 86% of the value attained at 30 min. Most of the asymptotic rise in the specific activity of ATP after 8 min could be accounted for by the incorporation of the label into α and β phosphate on the basis of a much slower rate of increase in the specific activity of ADP observed. It is therefore highly likely that γ phosphate of ATP has reached a steady state by 8 min. Addition of LPC at 10 min had no effect on the specific activity of ATP during the ensuing 20-min incubation period compared to the control without LPC.

Results

We examined the metabolism of LPC in 6C3HED, a mouse thymic lymphoma cell line, which we have previously reported

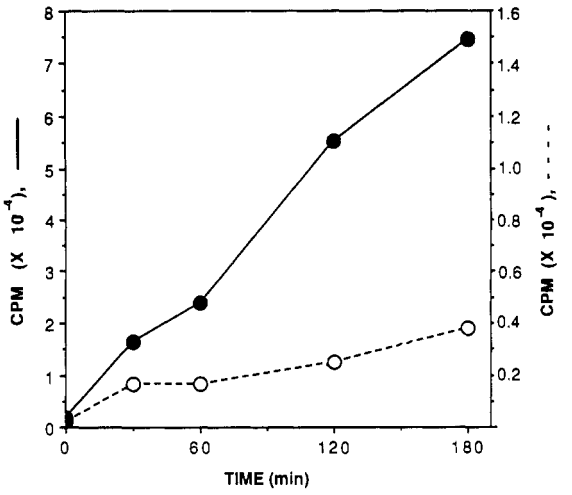

FIGURE 1: [^{14}C]FA-LPC metabolism in 6C3HED cells. Cells were incubated with [^{14}C]FA-LPC (50 μM final) at 37 °C, and lipids were extracted and analyzed as described under Experimental Procedures. Data are presented as cpm. The values shown on the left ordinate pertain to phosphatidylcholine (●) and those on the right ordinate to diacylglycerol (○). The mean ± SD of total cpm recovered from each sample was $1.22 \times 10^6 \pm 0.04 \times 10^6$. Similar results were obtained from two additional experiments.

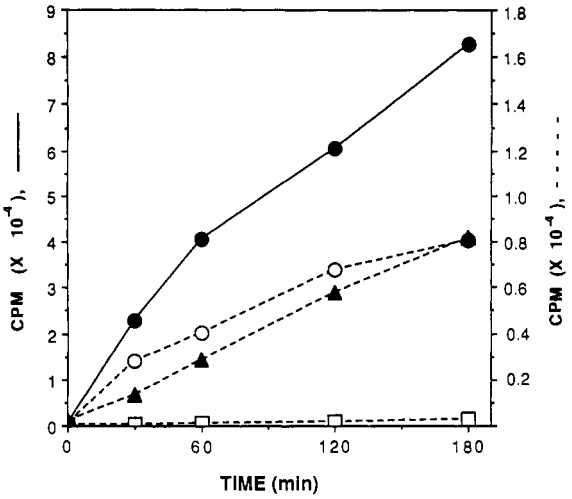

FIGURE 2: [^{14}C]Choline-LPC metabolism in 6C3HED cells. Cells were incubated with [^{14}C]choline-LPC (50 μM final) at 37 °C. Lipid and water-soluble products were extracted and analyzed as described under Experimental Procedures. Data are presented as cpm and are representative of results obtained in three experiments. The values shown on the left ordinate pertain to phosphatidylcholine (●) and those on the right ordinate to phosphocholine (○), glycerophosphocholine (▲), and choline (□). The total cpm recovered per sample was $1.04 \times 10^6 \pm 0.043 \times 10^6$ (mean ± SD). The value for choline at 180 min was 287 cpm.

to migrate in response to gradients of both LPC and cell-permeable analogues of DAG (Hoffman et al., 1982; Wright et al., 1988a). We were particularly interested in determining whether metabolic pathway(s) existed between LPC and DAG and studying the effect of DAG derived from LPC on protein kinase C activation.

Metabolism of Fatty Acid Labeled LPC. Cells from the mouse thymic lymphoma cell line, 6C3HED, were incubated with 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]-FA-LPC), 50 μM, for various lengths of time followed by extraction and analysis of neutral and phospholipid fractions as described under Experimental Procedures. There was a time-dependent incorporation of radioactivity into phosphatidylcholine and DAG (Figure 1). Also, the kinetics and the extent of radioactivity incorporation into free fatty acid (data not shown) were quite similar to those for phosphatidylcholine. In addition, we identified several minor radiolabeled products including phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, sphingomyelin, triacylglycerol, and monoacylglycerol, each representing less than 0.5% of total recovered counts after 2 h of incubation. Under these conditions the formation of radiolabeled DAG could occur via at least three pathways: first, phospholipase C mediated hydrolysis of phosphatidylcholine derived from LPC by acylation; second, phospholipase D mediated hydrolysis of phosphatidylcholine derived from LPC, followed by the action of phosphatidic acid phosphohydrolase; and third, the utilization of released [^{14}C]palmitic acid in de novo synthesis. To address this issue, we studied the metabolism of [1-^{14}C]palmitic acid to assess the contribution of ^{14}C-labeled fatty acid, released from [^{14}C]FA-LPC by lysophospholipase activity, in the formation of DAG and phosphatidylcholine. When 6C3HED cells were incubated in medium containing 50 μM [1-^{14}C]palmitic acid, radiolabel was readily incorporated into phosphatidylcholine, DAG, and phosphatidylethanolamine fractions. The incorporation of radiolabel into DAG was rapid during the first 30 min, but diminished thereafter (data not shown). These results indicate that a significant portion of labeled DAG and phosphatidylcholine formed from [^{14}C]FA-LPC may be due to incorporation of free fatty acid released from [^{14}C]FA-LPC by the action of lysolipase.

Metabolism of Choline-Labeled LPC. We next studied the metabolism of 1-palmitoyl-*sn*-glycero-3-phospho[N-*methyl*-^{14}C]choline ([^{14}C]choline-LPC) to determine whether there was a route of DAG formation from LPC independent of the pathway(s) involving liberated fatty acid. Cells were incubated in medium containing 50 μM [^{14}C]choline-LPC for 0 to 180 min, and after extraction, the lipid and water-soluble products were analyzed. We detected the formation of labeled phosphatidylcholine, glycerophosphocholine, phosphocholine, and trace amounts of free choline (0.03% of total recovered radioactivity after 3 h of incubation), as shown in Figure 2. Radiolabeled CDP-choline and sphingomyelin were not found. The results indicate that phosphatidylcholine was formed from LPC independent of the de novo synthetic pathway. The generation of phosphocholine suggested that phosphatidylcholine was hydrolyzed by phospholipase C.

Metabolism of Glycerol-Labeled LPC. To obtain direct evidence that LPC was metabolized to DAG independent of pathways involving liberated fatty acid, we prepared 1-palmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine ([^{14}C]glycerol-LPC). 6C3HED cells were incubated in medium containing 50 μM [^{14}C]glycerol-LPC for various time periods, and incorporated label was analyzed as described above. As shown in Figure 3, phosphatidylcholine was the major phospholipid product, and DAG was the major neutral lipid product. Analysis of the aqueous phases showed the presence of labeled glycerophosphocholine in amounts similar to that seen with [^{14}C]choline-LPC. The amounts of phosphatidylcholine generated from [^{14}C]choline-LPC and [^{14}C]glycerol-LPC were also comparable. In contrast, much less DAG was formed from [^{14}C]glycerol-LPC than predicted from the amount of phosphocholine formed from [^{14}C]choline-LPC. In addition, we detected 0.1% of total radioactivity in phosphatidylethanolamine but no significant formation of labeled phosphatidylserine, phosphatidylinositol, phosphatidic acid, monoacylglycerol, triacylglycerol, and glycerol (each less than 0.01% at 3 h). These results clearly demonstrate the conversion of LPC to phosphatidylcholine in 6C3HED cells and suggest that phosphatidylcholine was hydrolyzed to DAG by

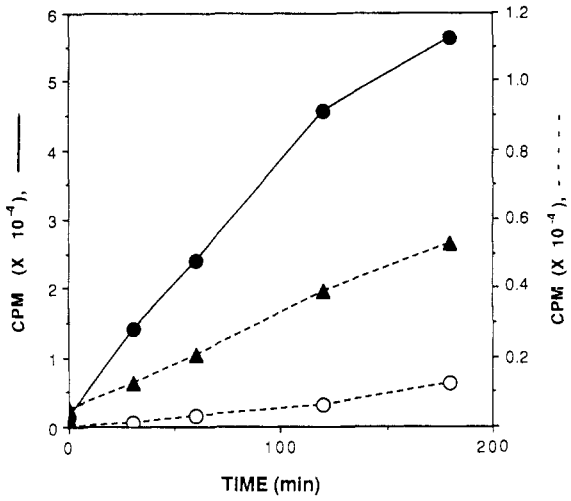

FIGURE 3: [^{14}C]Glycerol-LPC metabolism in 6C3HED cells. Cells were incubated with [^{14}C]glycerol-LPC (50 μM final) at 37 °C. Lipid and water-soluble products were extracted and analyzed as described under Experimental Procedures. Data are presented as cpm. The values shown on the left ordinate refer to phosphatidylcholine (●) and those on the right ordinate to glycerophosphocholine (▲) and diacylglycerol (○). The mean ± SD of total cpm recovered from each sample was $1.25 \times 10^6 \pm 0.02 \times 10^6$. Similar results were obtained in three additional experiments.

the action of a phospholipase C type enzyme. The possibility that LPC is first degraded to glycerol and that this glycerol contributed significantly to the formation of DAG is unlikely since excess (10 mM) cold glycerol had no effect upon metabolism of [^{14}C]glycerol-LPC to DAG. The discrepancy between the amounts of DAG and phosphocholine formed from glycerol-labeled and choline-labeled LPC, respectively, may in part be due to the rapid metabolism of DAG (Bishop & Bell, 1986), which would lead to a decrease in the activity recovered in this fraction.

Phosphatidylcholine-Hydrolyzing Phospholipase C in 6C3HED Homogenate. We next examined whether the phosphatidylcholine-hydrolyzing phospholipase C activity that was suggested by studies of LPC metabolism in intact cells could be identified in cell homogenates by using an exogenous substrate. Homogenates of 6C3HED cells were incubated with 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine at 37 °C followed by extraction and analysis of labeled products. As shown in Figure 4, phosphatidylcholine was hydrolyzed to DAG by cell homogenates. The initial velocity of this reaction was approximately 8 pmol per minute per milligram of crude homogenate protein ($\sim 10^7$ cells). There was no formation of DAG in control samples incubated without homogenate over a 2-h period (data not shown). The lipid extracts from these experiments were also analyzed by TLC for the production of polar lipid products (see Experimental Procedures). There was significant formation of LPC, indicating phospholipase A_2 activity in the cell homogenates. However, there was no detectable formation of labeled phosphatidic acid that would have suggested either hydrolysis of phosphatidylcholine by phospholipase D or phosphorylation of DAG by diacylglycerol kinase. We examined the possibility that the sequential action of phospholipase D and phosphatidate phosphohydrolase may contribute to the formation of DAG in 6C3HED homogenates. The addition of ethanol, 0.1–0.5% final concentration, previously demonstrated to reduce phosphatidic acid formation and lead to the generation of phosphatidylethanol by phospholipase D catalyzed transphosphatidylation (Eibl & Kovatchev, 1981; Bocckino et al., 1987; Gustavsson & Alling, 1987; Pai et al., 1988), failed to significantly reduce DAG

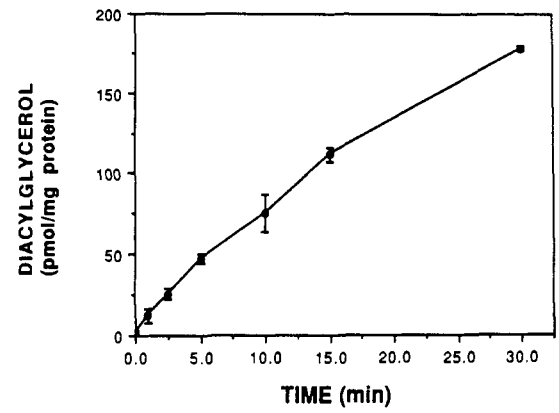

FIGURE 4: Diacylglycerol formation from 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine in 6C3HED homogenate. Cell homogenate was prepared and incubated with [^{14}C]glycerol-labeled phosphatidylcholine at 37 °C. Lipids were extracted and analyzed as described under Experimental Procedures. Results are expressed as picomoles of DAG per milligram of protein on the basis of the specific radioactivity of phosphatidylcholine. Each point represents the mean of duplicate determinations. Vertical bars denote the range. The data presented are representative of three separate experiments.

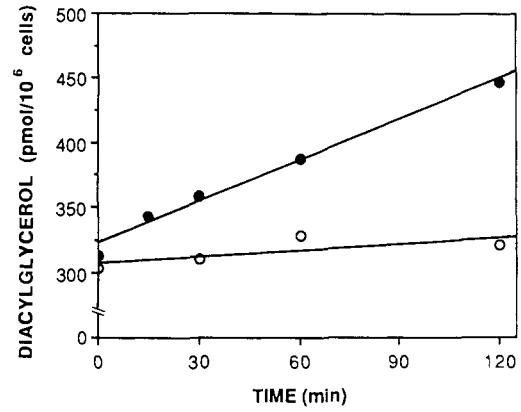

FIGURE 5: Effect of 1-palmitoyl-LPC on mass levels of DAG in 6C3HED cells. After washing in serum-free medium, 6C3HED cells were equilibrated at 37 °C for 30 min followed by the addition of either 1-palmitoyl-LPC (50 μM final) in serum-free medium (●) or serum-free medium alone (○). At the indicated times, lipids were extracted and the mass levels of DAG were determined as described under Experimental Procedures. The data are the means of three experiments, each performed in duplicate.

formation from 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine. In these experiments, labeled phosphatidylethanol was not formed by 6C3HED homogenates, but transphosphatidylation was readily demonstrated by using crude phospholipase D from cabbage (data not shown). We conclude that under the assay conditions described above the formation of DAG from radiolabeled phosphatidylcholine is the result of a phospholipase C. In other experiments, we incubated labeled LPC in the cell homogenate and failed to detect the formation of monoacylglycerol.

Mass Measurements of DAG in 6C3HED Cells. We incubated 6C3HED cells in medium containing 50 μM 1-palmitoyl-LPC or control medium for various time periods. Cellular lipids were extracted according to a modification of the methods of Bligh and Dyer (1959), and mass levels of DAG were determined by the diacylglycerol kinase assay (Preiss et al., 1986; Wright et al., 1988b). As shown in Figure 5, DAG mass levels rose steadily over a 2-h time course in cells incubated with LPC, reaching approximately 450 pmol/10^6 cells after 2 h. This represents an increase of 40% above cells incubated with control medium. We also examined the effect

Table I: Protein Phosphorylation in 6C3HED Cells Treated with LPC, DAG, and PMA[a]

treatment	phosphoproteins (M_r)		
	56 000	67 000	77 000
1-palmitoyl-LPC (50 μM)	129 ± 5[b]	132 ± 4[b]	138 ± 5[b]
DAG (1,2-dioctanoylglycerol, 25 μM)	127 ± 7[c]	141 ± 7[b]	143 ± 8[b]
PMA (10 nM)	120 ± 7[c]	134 ± 4[b]	138 ± 6[b]

[a] The incorporation of [^{32}P]orthophosphate into phosphoproteins was determined as described under Experimental Procedures. The data are expressed as the percent of ^{32}P incorporated in control cultures incubated with PFB alone. The values represent the means ±SE of 13 experiments. [b] $P < 0.001$. [c] $P < 0.01$.

of 2.5 μM palmitic acid on DAG mass levels in 6C3HED cells to investigate the possibility that the fatty acid liberated from LPC was responsible for the observed increase in DAG mass. This amount of palmitic acid was chosen because it represents the amount of free fatty acid estimated to be formed after 2 h from [^{14}C]FA-LPC on the basis of specific activity. Palmitic acid caused a transient 6% increase in DAG mass over control levels at 30 min; however, at 2 h the level of DAG in palmitic acid treated cells was increased <1% above control values.

Effect of LPC on Protein Phosphorylation. 6C3HED cells were prelabeled with [^{32}P]orthophosphate and incubated with LPC (50 μM), PMA (10 nM), or 1,2-dioctanoylglycerol (25 μM). The incorporation of label into phosphoproteins was performed as described under Experimental Procedures. LPC stimulated an increase in the phosphorylation of some proteins. This pattern of increased protein phosphorylation was also seen in cells stimulated with PMA or DAG (data not shown). An analysis of $^{32}P_i$ incorporation into several major phosphoproteins in response to LPC, PMA, and DAG is shown in Table I. All three stimuli induced a comparable increase in the phosphorylation of proteins with M_r 56 000, 67 000, and 77 000. The increase of protein phosphorylation induced by these three stimuli was selective in that a significant increase in phosphorylation was seen in some proteins but not in many others. Palmitic acid (2.5 μM final), which does cause a transient increase in DAG mass (see above), presumably by stimulating de novo synthesis, did not induce significant changes in the phosphorylation of these proteins (data not shown).

Discussion

The acylation of LPC has been studied in many cell types and tissues (Lands, 1960; Brumley & Van den Bosch, 1977; Chaudhary, 1982; Gross et al., 1983; Kramer et al., 1984). In lymphocytes, the activation of acyl CoA:LPC acyltransferase has been demonstrated following mitogen stimulation (Ferber & Resch, 1973; Ferber et al., 1975, 1976). Whereas these previous studies focused primarily on the acylation of LPC to phosphatidylcholine, little is known about the metabolic fate of this newly formed phosphatidylcholine. We investigated the metabolism of this phosphatidylcholine, derived from LPC, to neutral lipid and water-soluble products.

When 6C3HED cells were incubated with [^{14}C]FA-LPC, there was incorporation of radioactivity into three major lipid fractions: phosphatidylcholine, DAG, and free fatty acid. These experiments suggested a potentially important metabolic pathway between LPC and DAG. Additional metabolic studies were performed by using LPC labeled in the choline head group and glycerol backbone. The results of these experiments indicated that LPC was acylated to phosphatidylcholine and that a portion of this newly formed phosphatidylcholine was hydrolyzed to generate phosphocholine and DAG. The finding that phosphocholine rather than choline was formed suggested that phosphatidylcholine was hydrolyzed by a phospholipase C type enzyme rather than by the sequential action of phospholipase D and phosphatidate phosphohydrolase.

It is interesting that at all time points the amount of [^{14}C]phosphocholine recovered was 5–10-fold greater than the amount of [^{14}C]DAG. One possible explanation for this difference is that LPC could be hydrolyzed to monoglyceride and phosphocholine. However, our studies revealed no significant monoglyceride formation over a 3-h time course. It is unlikely that monoglyceride was not detected because of rapid degradation to glycerol and fatty acid, since glycerol and its immediate metabolites, glycerol 3-phosphate and phosphatidic acid, were also not formed in significant quantity. These intermediates were readily detected in amounts equivalent to or exceeding the level of labeled DAG in 6C3HED cells incubated with [^{3}H]glycerol (J.N., T.M.W., R.D.H., and H.S.S., unpublished data). These findings indicate that should this pathway of LPC metabolism contribute significantly to the formation of phosphocholine and DAG, it would have been readily apparent in the studies of [^{14}C]glycerol-LPC metabolism. In support of this conclusion, we failed to detect hydrolysis of LPC to phosphocholine and monoacylglycerol in cell homogenate. Also, excess (10 mM) cold glycerol had no effect on the conversion of [^{14}C]glycerol-LPC to DAG by 6C3HED cells. A more likely explanation for the difference in labeled phosphocholine and DAG is that these two products of phosphatidylcholine hydrolysis by phospholipase C are metabolized at different rates.

In several recent papers, the presence of a phospholipase C in mammalian cells capable of hydrolyzing phosphatidylcholine has been suggested by experiments using radiolabeled cells (Lockney et al., 1984; Besterman et al., 1986; Daniel et al., 1986; Muir & Murray, 1987; Irving & Exton, 1987; Takuwa et al., 1987; Rosoff et al., 1988). In this paper, we demonstrate phosphatidylcholine-hydrolyzing phospholipase C activity in homogenates of the lymphoblastic cell line 6C3HED. Soluble forms of phosphatidylcholine-hydrolyzing phospholipase C have been purified from bull seminal plasma (Sheikhnejad & Srivastava, 1986) and canine myocardium (Wolf & Gross, 1985). In contrast to these soluble enzymes, preliminary evidence indicates that the activity in 6C3HED cells, similar to that described by Martin et al. (1987) in bovine endothelial cells, resides in the particulate fraction of the cell and has a neutral pH optimum (T.M.W. and H.S.S., unpublished data). Further characterization of this activity awaits purification of the enzyme(s) involved.

LPC caused a steady rise in mass levels of DAG in 6C3HED cells. The magnitude of this increase in DAG mass is comparable to that reported in neutrophils stimulated with the chemotactic peptide, f-Met-Leu-Phe (Honeycutt & Neidel, 1986). The kinetics of the change in DAG mass levels in cells incubated with LPC were similar to the kinetics of labeled DAG formation observed in the metabolic studies. The amounts of DAG formed from LPC at 2 h, as determined by mass analysis and [^{14}C]FA-LPC metabolism, were 130 and 124 pmol/10^6 cells, respectively. Thus far, we have not detected any effects of LPC on phosphatidylinositol hydrolysis in 6C3HED cells (data not shown). These observations suggest that the increase in DAG mass caused by LPC was due to metabolism of LPC to DAG rather than the stimulation of DAG formation from other sources.

The formation of DAG from phosphatidylinositol following receptor-mediated cell stimulation has been associated with

the activation of protein kinase C in many cells [for review see Nishizuka (1986)]. An issue regarding the importance of DAG generated from sources other than inositol phospholipids is whether this DAG can also serve to activate protein kinase C. In 6C3HED cells treated with LPC, there was a substantial increase in the phosphorylation of many proteins. The pattern and magnitude of increased protein phosphorylation were similar to those observed in response to PMA and DAG (1,2-dioctanoylglycerol), two known activators of PKC (Nishizuka, 1984; 1986). These results suggest that DAG derived from LPC may be functionally important in cellular processes that involve the activation of protein kinase C.

Many ligands induce a transient hydrolysis of the polyphosphoinositides, generating inositol phosphates and DAG (Nishizuka, 1986; Bradford & Rubin, 1986; Rittenhouse & Sasson, 1985; Dillon et al., 1987). An important question that arises from this pattern of rapid and transient intracellular signals that follow receptor activation is whether these signals are responsible for cellular responses that are sustained (such as chemotaxis) or delayed (such as mitogenesis). Recent work from our laboratory as well as others has demonstrated the formation of DAG that does not parallel the breakdown of phosphatidylinositol in response to cell stimulation (Bocckino et al., 1985; Cockcroft & Allan, 1984; Raben et al., 1987; Kennerly, 1987; Wright et al., 1988b). In the present studies, LPC caused an increase both in the amount of labeled DAG and in the mass levels of DAG in 6C3HED cells that persisted for at least 2 h. It is possible, therefore, that pathways leading to DAG formation in addition to the hydrolysis of inositol phospholipids, such as the metabolism of LPC to DAG, may be important in the transduction of signals that evoke sustained or delayed cellular responses.

In conclusion, the studies of exogenous LPC metabolism by intact 6C3HED cells and phosphatidylcholine metabolism by cell homogenates provide additional evidence for the hydrolysis of phosphatidylcholine by phospholipase C. These studies further suggest that endogenous LPC, generated by phospholipase A_2 which can be activated by receptor stimulation (Hong & Deykin, 1982; Bormann et al., 1984; McKean et al., 1981), may participate in cell activation through its metabolism to DAG with subsequent stimulation of protein kinase C.

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Registry No. Choline-labeled LPC, 77165-67-4; labeled LPC, 119336-75-3; phospholipase C, 9001-86-9; 1,2-dipalmitoyl-*sn*-glycero-3-phospho[N-methyl-^{14}C]choline, 119336-70-8; 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*,*N*-dimethylethanolamine, 3922-61-0; L-[U-^{14}C]glycerol 3-phosphate, 119336-71-9; L-[U-^{14}C]glycerol 3-phosphate pyridine salt, 65114-65-0; palmitic anhydride, 623-65-4; 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycerol 3-phosphate (dimethylamino)-pyridinium salt, 119336-73-1; choline tetraphenylborate, 75667-82-2; 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycero-3-phosphocholine, 119336-74-2; palmitic acid, 57-10-3; glycerophosphocholine, 28319-77-9; phosphocholine, 107-73-3.

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Platelet Receptor Recognition Domains on the α Chain of Human Fibrinogen: Structure–Function Analysis†

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ABSTRACT: We have previously shown that the α chain of human fibrinogen interacts directly with ADP-activated human platelets [Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D. D., & Doolittle, R. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2068]. Now, we report that platelet receptor recognition domains are localized on two CNBr fragments of the human fibrinogen α chain. They encompass residues 92–147 and 518–584, which inhibit ^{125}I-fibrinogen binding to ADP-stimulated platelets. The inhibitory CNBr fragment α92–147 contains the RGD sequence at residues 95–97. Synthetic peptides encompassing this sequence were inhibitory while peptide 99–113 lacking the RGD sequence was inactive. The synthetic peptide RGDF, corresponding to residues α95–98, inhibited the binding of ^{125}I-fibrinogen to ADP-treated platelets (IC_{50} = 2 μM). However, the peptides containing sequence RGDF, with residues preceding Arg^{95} or following Phe^{98}, were less inhibitory. It appears that the sequence α95–98 constitutes a platelet receptor recognition domain which is constrained by flanking residues. The second inhibitory CNBr fragment, α518–584, also contains the sequence RGD at positions 572–574. Synthetic peptides overlapping this sequence were inhibitory, while peptides lacking the sequence RGDS were not reactive. Thus, another platelet reactive site on the α chain encompasses residues 572–575 containing sequence RGDS. In conclusion, the platelet receptor recognition domains on the human fibrinogen α chain in the amino-terminal and in the carboxy-terminal zones contain the ubiquitous cell recognition sequence RGD shared with other known adhesive proteins. Together with a unique domain at the carboxy-terminal end of the γ chain, they endow human fibrinogen with six sites for interaction with activated platelets.

Human fibrinogen, a clottable and adhesive protein, is composed of three pairs of nonidentical chains (α, β, γ) linked by a series of disulfide bonds and arranged in three main structural domains: one central E and two distal D (Doolittle, 1984). We showed that both the γ chain and the α chain bear sites interacting with receptors on ADP-activated platelets (Hawiger et al., 1982). The γ-chain site has been subsequently pinpointed to the last 12 carboxy-terminal residues (Kloczewiak et al., 1984). Structure–function analysis of modified peptides patterned on the carboxy-terminal segment, γ 400–411, indicates that the continuous 12 amino acid sequence is required for optimal reactivity toward platelet receptors [see second of three papers in this issue (Kloczewiak et al., 1989)].

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