Biochemical Pharmacology, Vol. 46, No. 1, pp. 139–148, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ASSOCIATION BETWEEN LEUKOTRIENE B_4-INDUCED PHOSPHOLIPASE D ACTIVATION AND DEGRANULATION OF HUMAN NEUTROPHILS

HAN-LIANG ZHOU,* MARIE CHABOT-FLETCHER, JAMES J. FOLEY, HENRY M. SARAU, MARITSA N. TZIMAS, JAMES D. WINKLER and THEODORE J. TORPHY†

Department of Inflammation and Respiratory Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406-0939, U.S.A.

(*Received* 25 *August* 1992; *accepted* 1 *March* 1993)

Abstract—We have explored the role of phospholipase D (PLD) activation in leukotriene B_4 (LTB_4)-induced Ca^{2+} mobilization and degranulation of human neutrophils. Stimulation of [^{3}H]alkyl-acyl-phosphatidylcholine-labeled neutrophils with LTB_4 resulted in a rapid accumulation of [^{3}H]alkyl-phosphatidic acid (PA) as well as a somewhat slower accumulation of [^{3}H]alkyl-diglyceride (DG). In the presence of ethanol, PLD catalyzed a transphosphatidylation reaction in which LTB_4 increased [^{3}H]alkyl-phosphatidylethanol formation and simultaneously decreased LTB_4-induced PA and DG accumulation. This pattern of lipid metabolism is consistent with the conclusion that LTB_4 stimulates PLD activity in human neutrophils. Additional studies in which the extracellular and intracellular concentrations of Ca^{2+} were varied indicated that maximal LTB_4-induced PLD activation was dependent upon Ca^{2+} and potentiated by inhibitors of protein kinase C. The time–course and concentration–response curves for LTB_4-induced PLD activation were different from those for LTB_4-induced Ca^{2+} mobilization, as measured by fura-2 fluorescence. On the other hand, the concentration–response curve for LTB_4-induced PLD activation was similar to that for LTB_4-induced degranulation. Preincubation of the cells with ethanol inhibited LTB_4-induced PA and DG accumulation, as well as degranulation, suggesting that one or both of these metabolites were important for this response. In contrast, ethanol had no effect on LTB_4-induced Ca^{2+} mobilization. Propranolol, an inhibitor of phosphatidate phosphohydrolase, abolished DG accumulation in response to LTB_4 but had no effect on degranulation, suggesting that PA is more important than DG as a mediator of degranulation. Taken collectively, these data indicate that LTB_4-induced activation of PLD in human neutrophils is mediated by a Ca^{2+}-dependent mechanism, but not by protein kinase C. In addition, PLD activation in these cells may induce degranulation, but not Ca^{2+} mobilization.

Leukotriene B_4 (LTB_4‡) is a 5-lipoxygenase product that stimulates chemotaxis, aggregation and degranulation of neutrophils and other inflammatory cells [1]. As a prerequisite for producing these functional effects, LTB_4 activates a variety of enzymes, including phospholipase C (PLC) and protein kinase C (PKC) [2–5], and induces Ca^{2+} mobilization [6–8]. In addition, it has been reported recently that LTB_4 activates phospholipase D (PLD) in rabbit and human neutrophils [9, 10]. PLD catalyzes the hydrolysis of phospholipids, primarily choline-containing phosphoglycerides (phosphatidylcholine, PC), to generate phosphatidic acid (PA) and choline. PA can be degraded further by phosphatidate phosphohydrolase to yield diacyl- and alkylacylglycerols (DG), which may then activate PKC. PA may also have a second messenger role in promoting Ca^{2+} entry into cells [11–14], mobilizing intracellular Ca^{2+} [15, 16], inhibiting adenylyl cyclase [16–18], activating phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific PLC [19], stimulating DNA synthesis and cell proliferation [15, 20], regulating stimulus–secretion coupling [21–23], and activating NADPH oxidase [24, 25]. Although PLD has thus been implicated indirectly in mediating a number of cellular processes, limited evidence is available that strongly links activation of PLD with functional responses [25–30].

The present studies were carried out in an effort to determine whether a link exists between LTB_4-induced PLD activation and key biological functions of the human neutrophil. The results provide evidence to support a role for PLD and its product, PA, in mediating LTB_4-induced degranulation of this cell, but not a role in stimulating Ca^{2+} mobilization.

MATERIALS AND METHODS

Materials. 1-[*alkyl*-1′,2′-^{3}H]*sn*-Glyceryl-3-phosphorylcholine (40 Ci/mmol) was purchased from

* Present address: Department of Pharmacology, Zhejiang Medical University, Hangzhou 310006, China.

† Corresponding author: Dr Theodore J. Torphy, Department of Inflammation and Respiratory Pharmacology, L532, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406-0939. Tel. (215) 270-6821; FAX (215) 270-5381.

‡ Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DG, 1-radyl-2-acyl-glycerol (diglyceride); EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; KRH, Krebs–Ringer–Henseleit buffer; LTB_4, leukotriene B_4; MPO, myeloperoxidase; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidylethanol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; and PMA, phorbol 12-myristate-13-acetate.

New England Nuclear (Boston, MA). Phorbol 12-myristate-13-acetate (PMA), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), cytochalasin B, staurosporine, histopaque-1077, calphostin C and (±)-propranolol were obtained from Sigma (St. Louis, MO). Essentially fatty acid-free bovine serum albumin was obtained from Calbiochem (San Diego, CA). LTB_4 and Ly 223982 were synthesized by the Department of Medicinal Chemistry (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). Chelerythrine was obtained from LC Services (Woburn, MA), and R55949 was supplied by Dr. D. de Chaffroy de Courcelles, Janssen Research Foundation (Beerse, Belgium). Precoated silica gel 60 plates (0.25 mm thick) were purchased from E. Merck (Darmstadt, Germany). PMA, A23187, fMLP, cytochalasin B, staurosporine, chelerythrine and Ly 223982 were dissolved as 10 mM stock solutions in dimethyl sulfoxide before further dilution into Krebs–Ringer–Henseleit buffer (KRH). The final concentration of dimethyl sulfoxide did not exceed 0.2% and did not affect the parameters being measured. LTB_4 stock solutions (2 mM) were prepared in a methanol:H_2O (3:1) mixture before being further diluted into KRH. Other drugs were prepared as aqueous solutions.

Preparation of human neutrophils. Human neutrophils were isolated from freshly drawn heparinized venous blood obtained from healthy donors by the procedure of Böyum [31]. Briefly, human blood was layered over Histopaque-1077, and the pellets were collected after centrifugation (400 *g*, 20 min at room temperature), resuspended and sedimented with 1.5% dextran for 45 min. The resulting pellets were exposed briefly to deionized water to lyse erythrocytes. The remaining cells were collected by centrifugation, suspended in buffer (137 mM NaCl, 2.7 mM KCl, 8.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4), and counted. All final leukocyte preparations were of greater than 95% purity and viability, as determined by histological examination and trypan blue exclusion, respectively.

Radiolabeling of human neutrophils. Purified neutrophils were resuspended to a density of 3.3×10^7/mL in KRH (118 mM NaCl; 4.6 mM KCl; 24.9 mM $NaHCO_3$; 1 mM KH_2PO_4; 11.1 mM D-glucose; 1 mM $CaCl_2$; 1.1 mM $MgCl_2$; 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 1 mg/mL radioimmunoassay grade fatty acid-free bovine serum albumin; pH 7.4). Cellular 1-alkyl-PC pools were radiolabeled by incubating cells with 5 μCi/mL of 1-[*alkyl*-1′,2′-^{3}H]-*sn*-glyceryl-3-phosphocholine for 60 min at 37°. Preliminary studies demonstrated that >95% of the radiolabel had been taken up by the cells at this time and >90% of the cell-associated radioactivity was 1-alkyl-PC [32]. After labeling, unincorporated radioactivity was removed by washing cells twice with KRH. Washed, radiolabeled neutrophils were then suspended in KRH at a concentration of 10^7 cells/mL and used to measure PLD activity.

PLD assay. Measurement of [^{3}H]alkyl-PA and [^{3}H]alkyl-DG was used as an indirect index of changes in the mass of PA and DG. Labeled neutrophils (5×10^6) were prewarmed for 5 min at 37° before being exposed to vehicle, LTB_4 or other agonists (PMA and fMLP). Incubations were carried out in the absence or presence of Ca^{2+}, ethanol, propranolol or various PKC inhibitors (staurosporine, chelerythrine, calphostin C) for the indicated time. Samples with calphostin C were incubated under direct light. All experiments were performed in the presence of 5 μM cytochalasin B except where noted in the figure legends. At the appropriate time, the reaction was stopped by adding 3 vol. of chloroform:methanol:acetic acid (100:200:4, by vol.). The lipids were then extracted by the procedure of Bligh and Dyer [33]. An aliquot (typically 100,000–500,000 dpm) of the lower chloroform phase was dried and redissolved in chloroform, and the lipids were separated by thin-layer chromatography on silica gel 60 plates. None of the drug treatments altered the total amount of radioactivity extractable from the cell preparations. Plates were developed using the organic phase of ethyl acetate:iso-octane:acetic acid:water (110:50:20:100, by vol.). Lipid standards were used to identify the separated lipid fractions [PC, $R_f = 0$; PA, $R_f = 0.09$; phosphatidylethanol (PEt), $R_f = 0.15$; monoglycerides, $R_f = 0.5$; DG, $R_f = 0.91$; triglycerides, $R_f = 0.97$] [34]. The [^{3}H] lipid products were located by scanning individual lanes with a Bioscanner (Imaging Scanner 200-IBM, Washington, DC), and the silica gel areas containing individual lipids were scraped and the radio activity was quantified by liquid scintillation spectrometry. Data were expressed as a percentage of the total radioactivity applied to the plate.

Calcium mobilization methods. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was estimated using the Ca^{2+} fluorescent probe fura-2. Isolated neutrophils were suspended in KRH at a concentration of 2×10^6/mL and incubated with 2 μM fura-2/AM, the pentaacetoxymethyl ester of fura-2, at 37° for 45 min. Cells were centrifuged at 225 *g* for 10 min, resuspended in KRH buffer, and incubated at 37° for an additional 15 min to allow complete hydrolysis of the intracellular fura-2 ester. Neutrophils were centrifuged, resuspended at 4×10^6 cells/mL and maintained at room temperature until used for fluorescence determination, which was performed within 2 hr. Prior to fluorescence determination the cells were diluted to a final concentration of 10^6 cells/mL in KRH and warmed to 37°. Aliquots (2 mL) of the cell suspension were added to a 1-cm^2 cuvette and placed into a fluorometer (Johnson Foundation Biomedical Instrumentation Group, Philadelphia, PA), equipped with a temperature control and a magnetic stirrer under the cuvette holder. The excitation filter was 340 nm (10 nm band width) and the emission filter was 510 nm (20 nm band width). Fluorescence was recorded for 1 min to ensure a stable baseline before addition of agonist. Fluorescence was recorded continuously for at least 2 min after agonist addition. The $[Ca^{2+}]_i$ was calculated using methods described previously [35]. Data are presented as maximal agonist-induced increases in $[Ca^{2+}]_i$ over baseline (prestimulated levels).

Degranulation assay. Degranulation was assessed using 10^6 neutrophils in a final volume of 1 mL. Neutrophils were preincubated with 5 μg/mL

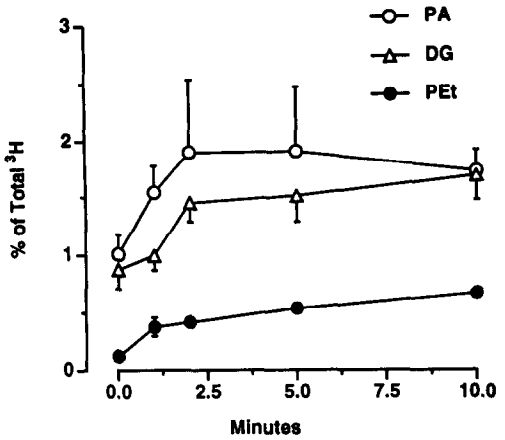

Fig. 1. Time-course of LTB_4-induced accumulation of PA, DG and PEt in human neutrophils. [^{3}H]Alkyl-acyl-PC-prelabeled cells were incubated with 1 μM LTB_4 in the presence (●) or absence (○, △) of 0.5% ethanol for the times indicated. PA, DG and PEt were separated by TLC, and radioactivity was quantified by scintillation counting. Data are the means ± SEM of 3 experiments.

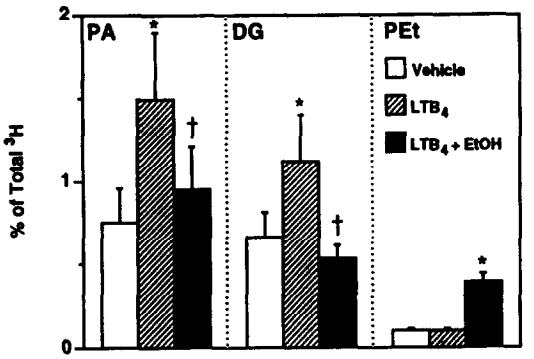

Fig. 2. Effect of ethanol on LTB_4-induced PA, DG and PEt production in human neutrophils. [^{3}H]Alkyl-acyl-PC-prelabeled cells were incubated with KRH (open bars) or 1 μM LTB_4 in the presence (solid bars) or absence (hatched bars) of 0.5% ethanol (EtOH) for 2 min. PA, DG and PEt were separated by TLC, and radioactivity was quantified by scintillation counting. Data are the means ± SEM of 4–5 experiments. Key: (*) greater than control ($P < 0.05$), and (†) less than in the presence of LTB_4 alone ($P < 0.05$).

cytochalasin B for 10 min at 37° followed by LTB_4 for 3 min. Incubations were conducted in the absence or presence of Ca^{2+}, ethanol, staurosporine, propranolol or Ly 223982 added 5 min before agonist challenge. In time-course studies, incubations were terminated at specific times by separating the extracellular medium from the neutrophils by drawing the suspended cells through a syringe equipped with a 0.45 μm HV filter (Millipore, Bradford, MA). In all other studies, the incubation was terminated by placing assay tubes on ice followed by centrifugation (400 *g* for 2 min). Myeloperoxidase (MPO) activity in the supernatant fractions was determined kinetically as described by Bradley *et al.* [36]. Briefly, 50 μL of the supernatant was incubated with 0.95 mL assay reagent containing 0.167 mg/mL *O*-dianisidine dihydrochloride, 0.0005% hydrogen peroxide and 50 mM potassium phosphate buffer (pH 6.0). Product formation was linear for >2 min and measured spectrophotometrically every 15 sec throughout a 2-min period in a Beckman DU-70 spectrophotometer at 460 nm. One unit of MPO activity was defined as that degrading 1 μmol of peroxide/min at 25°.

Data analysis and statistical evaluation. Each set of experiments was performed 3–5 times using cells from different donors. The data are expressed as means ± SEM. Differences between two means were determined by a paired Student's *t*-test and accepted as significant at $P < 0.05$.

RESULTS

Effect of LTB_4 on PLD activity, Ca^{2+} mobilization and degranulation. Initial experiments were carried out to assess the ability of LTB_4 (1 μM) to stimulate the production of PA, DG and, in the presence of ethanol, PEt in human neutrophils. In these and all subsequent experiments PA and DG were measured as [^{3}H]alkyl-PA and [^{3}H]alkyl-DG, respectively. As shown in Fig. 1, the production of these products in response to LTB_4 was time dependent. It is noteworthy that PA accumulation appeared to precede the appearance of DG and that the levels of PEt had increased more than 3-fold by 1 min. By comparison, LTB_4-induced Ca^{2+} mobilization was extremely rapid, reaching a maximum within 8–10 sec (see Fig. 8), and thus appeared to precede the formation of PA, DG and PEt. In other experiments, MPO release was increased within 30 sec of exposure to 1 μM LTB_4 (0.013 units/10^6 cells, control vs 0.035 units/10^6 cells, plus LTB_4; N = 2) and PA accumulation doubled within the same period (1.0%, control vs 1.9%, plus LTB_4; N = 2).

Ethanol serves as an alternative substrate to H_2O in the PLD-catalyzed hydrolytic cleavage of choline from PC. Thus, ethanol, rather than a hydroxyl group from H_2O, is incorporated via a trans-phosphatidylation reaction into the *sn*-3 position of PC to form PEt. Theoretically, then, formation of PEt in the presence of ethanol should be accompanied by a decrease in the formation of the normal products of PLD, PA and its hydrolytic metabolite, DG. Indeed, in the presence of 0.5% ethanol, the ability of 1 μM LTB_4 to stimulate the production of PEt was accompanied by an inhibition of DG and PA generation (Fig. 2). Because of the reciprocal relationship between the accumulation of PEt vs PA and DG, and because the production of PEt is a unique property of PLD, these results support the concept that LTB_4-induced accumulation of PA and DG is mediated by PLD activation. Moreover, a preliminary experiment indicated that 3 μM R 55949, a DG kinase inhibitor [37], had no effect on LTB_4-induced PA and DG accumulation, suggesting that PLC activation was not involved with the response.

PLD activity, Ca^{2+} mobilization and degranulation were stimulated by LTB_4 in a concentration-dependent manner (Fig. 3). LTB_4 stimulated PLD activity and degranulation over a similar concentration range, but was approximately 10-fold more potent in stimulating Ca^{2+} mobilization. Thus,

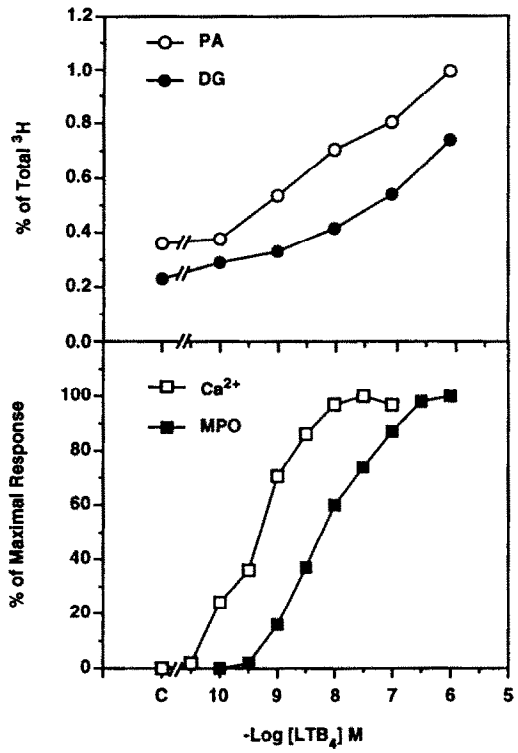

Fig. 3. Degranulation, Ca^{2+} mobilization, and PA and DG accumulation in response to various concentrations of LTB_4 in human neutrophils. PA and DG were measured after a 2-min exposure to LTB_4. Degranulation measured as MPO release was determined after a 3-min exposure to LTB_4. Ca^{2+} mobilization represents the peak response to LTB_4 (10–15 sec after LTB_4 administration). In the experiments shown, maximal MPO release in response to 1 μM LTB_4 was 4.3×10^{-2} units/10^6 cells and the basal $[Ca^{2+}]_{cyt}$ of 180 nM was increased by 30 nM LTB_4 to 1077 nM. Each point is the mean of duplicate determinations from a single representative of 3 experiments.

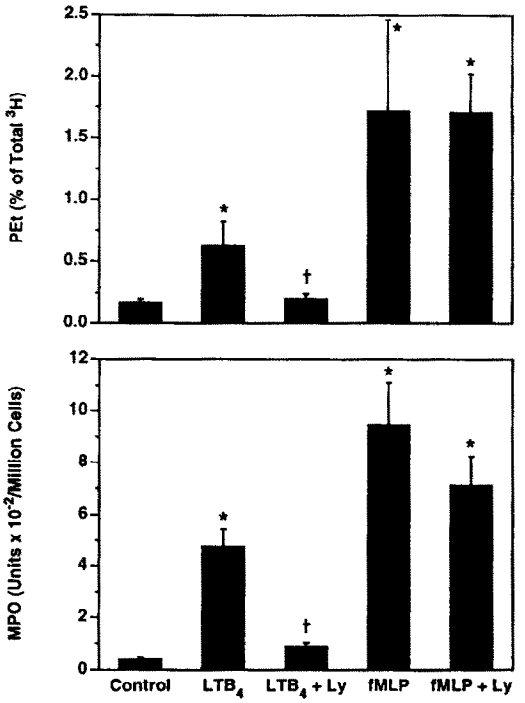

Fig. 4. Effect of Ly 223982 on LTB_4- and fMLP-induced PLD activation and MPO release in human neutrophils. Cells were exposed to vehicle or 10 μM Ly 223982 (Ly) for 5 min before being challenged with 1 μM LTB_4 or 1 μM fMLP. Formation of PEt (top panel) and release of MPO (bottom panel) were determined 5 and 3 min, respectively, after agonist addition. Data are the means ± SEM of 4 experiments. Key: (*) significantly greater than control value ($P < 0.05$), and (†) significantly less than value in the presence of agonist alone ($P < 0.05$).

at LTB_4 concentrations that elicited a maximal increase in Ca^{2+} mobilization (i.e. 10 nM LTB_4), PLD activity and degranulation were stimulated to only one half of their maximal values.

In another study, pretreatment of cells with 10 μM Ly 223982, an LTB_4-receptor antagonist [38], abolished PEt production and degranulation in response to 1 μM LTB_4 (Fig. 4). In contrast, Ly 223982 had no significant effect on responses to 1 μM fMLP, thus demonstrating the selectivity of the antagonist for LTB_4. These results confirm that the ability of LTB_4 to produce its functional effects in the neutrophil is mediated by its interaction with the LTB_4 receptor.

Effect of cytochlasin B. Previous studies have demonstrated that cytochalasin B increases PLD activity in response to receptor-mediated agonists [9, 39, 40]. To extend this observation to the action of LTB_4, we investigated the effect of cytochalasin B on LTB_4-induced PLD activation, degranulation and Ca^{2+} mobilization in human neutrophils. Pretreatment of cells with cytochalasin B (5 μM) markedly potentiated the accumulation of PA and PEt, as well as MPO release, induced by 1 μM LTB_4 by 4-, 4- and 17-fold, respectively (Fig. 5). Cytochalasin B also increased Ca^{2+} mobilization in response to a supramaximal concentration of LTB_4 (100 nM), although the increase was less than 2-fold.

Effect of ethanol on LTB_4-induced PLD activity, degranulation and Ca^{2+} mobilization. The effect of ethanol on LTB_4-induced PEt, PA and DG accumulation, as well as MPO release and Ca^{2+} mobilization, is shown in Fig. 6. Increasing the concentration of ethanol over a range of 0 to 1% resulted in a concentration-dependent increase in PEt formation and a concomitant inhibition of LTB_4-induced PA and DG formation as well as MPO release. In contrast, ethanol had no effect on Ca^{2+} mobilization in response to LTB_4. These data suggest that LTB_4-induced PLD activation is functionally linked to degranulation, but not to Ca^{2+} mobilization, in human neutrophils.

Effect of propranolol on LTB_4-induced degranulation. To determine which product of PLD might be responsible to degranulation, we studied the effects of propranolol, an inhibitor of phosphatidate phosphohydrolase, on the functional responses of neutrophils to LTB_4. Figure 7 shows that 200 μM propranolol abolished DG production and potentiated PA production in response to a maximal concentration of LTB_4 (1 μM). In spite of the abolition of LTB_4-induced DG accumulation by propranolol, LTB_4-induced degranulation was not inhibited in the presence of propranolol, suggesting

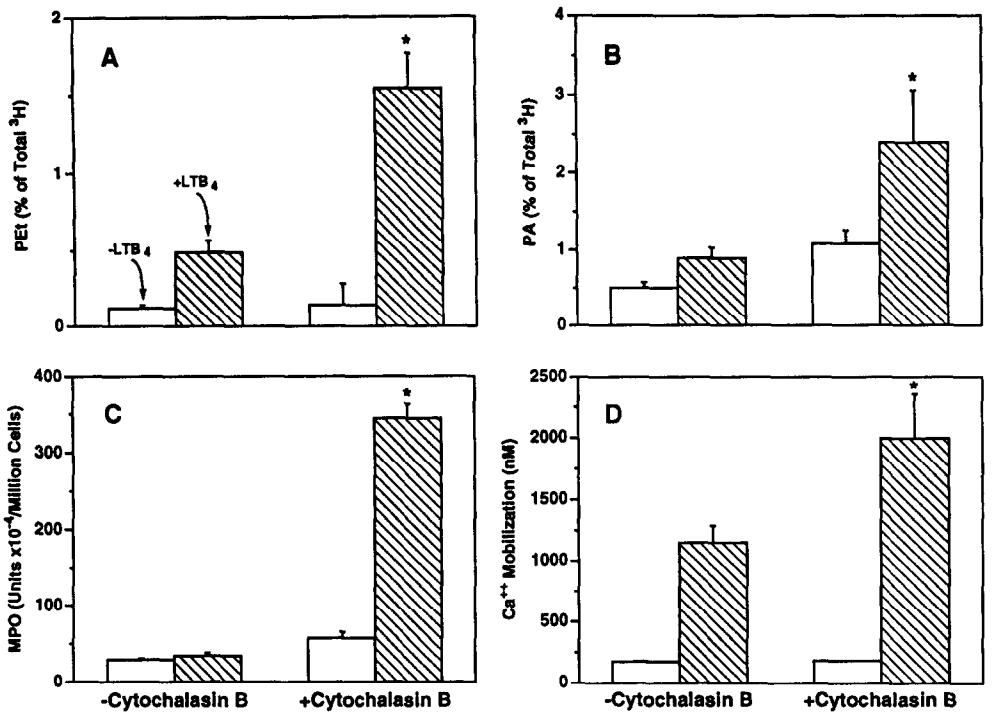

Fig. 5. Effect of cytochalasin B on LTB_4-induced PEt and PA accumulation, degranulation and Ca^{2+} mobilization in human neutrophils. PEt (panel A) was measured 15 min after exposure to 1 μM LTB_4. PA (panel B) was measured 2 min after exposure to 1 μM LTB_4. MPO release (panel C) was measured 3 min after exposure to 1 μM LTB_4. Ca^{2+} mobilization (panel D) was measured 10–15 sec after exposure to 100 nM LTB_4. In every panel, the two bars on the left represent data from cells pretreated with vehicle and the two bars on the right represent data from cells pretreated with 5 μM cytochalasin B. The open bars represent the basal values and the hatched bars represent LTB_4-stimulated values. Data are the means ± SEM of 3–4 experiments. Key: (*) greater than the LTB_4-stimulated value in the absence of cytochalasin B ($P < 0.05$).

that PA rather than DG is the PLD product that is involved in LTB_4-induced degranulation. Propranolol also failed to potentiate degranulation in response to a wide range of LTB_4 concentrations (1 nM–1 μM) (data not shown), even though it potentiated LTB_4-induced PA accumulation.

Role of Ca^{2+} *and PKC.* Multiple mechanisms are thought to be involved in mediating receptor-coupled PLD activation [41]. In many cell types, phorbol esters and Ca^{2+} ionophores activate PLD [32, 42], suggesting that both PKC- and Ca^{2+}-dependent pathways are linked to PLD activation. To explore the role of PKC and Ca^{2+} in LTB_4-induced PLD activation in neutrophils, the effect of changing extracellular and intracellular Ca^{2+} concentrations on LTB_4-induced PLD activation and degranulation was investigated, as were the effects of PKC inhibitors.

To investigate the effect of Ca^{2+} on the activation of PLD and neutrophil degranulation, Ca^{2+} stores were depleted with the Ca^{2+}-chelator ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). The importance of extracellular Ca^{2+} was studied as follows. Neutrophils were resuspended in Ca^{2+}-free KRH and 10 sec prior to stimulation with LTB_4, EGTA (200 μM) was added to the cell suspension to remove extracellular Ca^{2+}. As determined by Ca^{2+} levels during the post-stimulus plateau phase of the response, Ca^{2+} influx was virtually abolished by 200 μM EGTA and total Ca^{2+} mobilization was reduced by 75% (Fig. 8, A and B). Under these conditions, PLD activation and degranulation in response to LTB_4 were decreased by 72 and 45%, respectively (Fig. 9), suggesting that Ca^{2+} influx contributed to maximal LTB_4-induced PLD activation and degranulation.

The role of intracellular Ca^{2+} was investigated further by incubating neutrophils in Ca^{2+}-free KRH containing 50 μM EGTA for 30 min. This treatment removed extracellular Ca^{2+} and depleted most of the intracellular Ca^{2+} stores, as evidenced by the ability of LTB_4 to produce only a small, transient Ca^{2+} signal in these cells (Fig. 8C). Depleting both intra- and extracellular Ca^{2+} abolished LTB_4-induced PLD activity and produced a further, substantial reduction in degranulation (Fig. 9). Re-exposing cells to 1 mM Ca^{2+} after they had been treated with 50 μM EGTA for 30 min returned their ability to respond to LTB_4 nearly to the same level as untreated cells (Figs. 8D and 9). These results suggest that in neutrophils both LTB_4-stimulated PLD activity and degranulation are dependent on the mobilization of intracellular Ca^{2+} as well as its influx from extracellular stores.

The role of PKC in LTB_4-stimulated PLD activation and degranulation was investigated by incubating neutrophils with the kinase inhibitor staurosporine (1 μM) prior to stimulation with LTB_4.

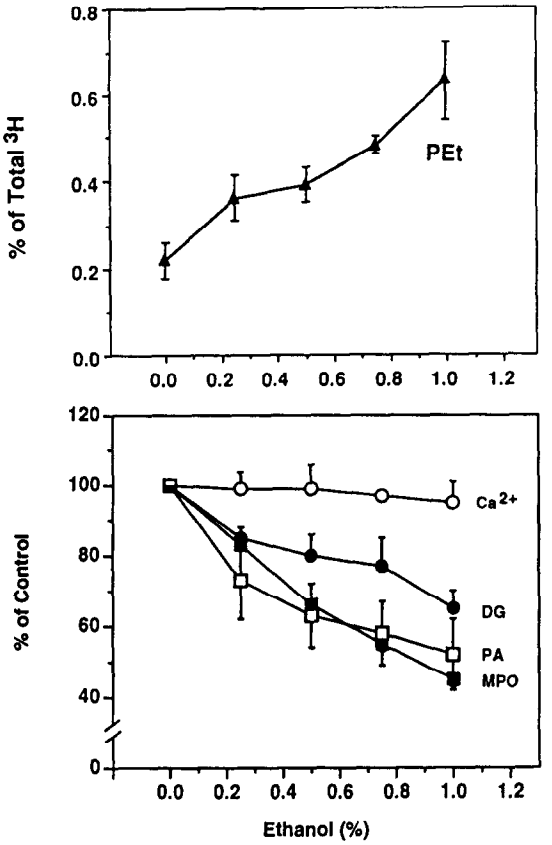

Fig. 6. Effect of ethanol on LTB_4-induced PEt, PA and DG accumulation, degranulation and Ca^{2+} mobilization in human neutrophils. The parameters were quantitated in the presence of 0, 0.25, 0.5, 0.75 or 1% ethanol. PEt (▲), PA (□) and DG (●), MPO release (■) and Ca^{2+} mobilization (○) were measured 2 min, 2 min, 2 min, 3 min or 10–15 sec, respectively, after exposure to 1 μM (PEt, PA, DG and MPO) or 100 nM (Ca^{2+} mobilization) LTB_4. PEt data (top) is expressed as a percentage of total ^{3}H. The remaining data (bottom) are expressed as the percentage of the values obtained in the absence of ethanol (PA, 1.02 ± 0.09% total ^{3}H; DG, 1.22 ± 0.40% total ^{3}H; MPO, 0.038 ± 0.0007 units/10^6 cells; and Ca^{2+}, 773 ± 105 nM above baseline). Significant decreases in PA, DG and MPO were observed at both 0.75 and 1.0% ethanol ($P < 0.05$; ANOVA with Scheffe F analysis of original data). Each point is the mean ± SEM of 3–4 experiments.

Such treatment did not significantly alter basal PLD activity or MPO release, but potentiated LTB_4-stimulated PLD activation and degranulation (Fig. 10). In contrast, PEt accumulation in response to 100 nM PMA (0.16% of total ^{3}H, control 9.4% of total ^{3}H, PMA) was reduced significantly (6.1% of total ^{3}H), but not abolished, by 1 μM staurosporine ($N = 2$). Two other PKC inhibitors, 5 μM chelerythrine [43] and 1 μM calphostin C [44, 45], also potentiated LTB_4-induced PLD activation (Table 1). Thus, the activation of PKC does not appear to mediate PLD activation and degranulation of neutrophils in response to LTB_4.

DISCUSSION

Although the ability of LTB_4 to stimulate PLD activity in human neutrophils has been reported previously [10, 46], the mechanism and physiological significance of LTB_4-induced PLD activation are not completely understood. In this study, we investigated: (1) the characteristics of LTB_4-induced PLD activation in human neutrophils; (2) the mechanism by which LTB_4 activates PLD; and (3) the role of PLD activity in degranulation and Ca^{2+} mobilization.

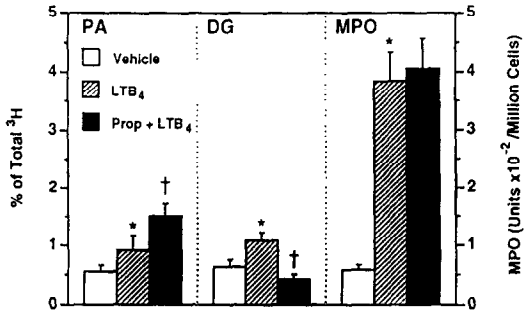

Fig. 7. Effect of propranolol on LTB_4-induced degranulation and the production of PA and DG in human neutrophils. [^{3}H]Alkyl-acyl-PC-prelabeled cells (for PA and DG production) and non-prelabeled cells (for MPO release) were incubated with KRH (open bars) or 1 μM LTB_4 for 5 min (PA and DG) or 3 min (MPO) in the absence (hatched bars) or presence (solid bars) of 200 μM propranolol. PA and DG were separated by TLC, and radioactivity was quantified by scintillation counting. MPO activity was assessed as described in Materials and Methods. Data are the means ± SEM of 4–5 experiments. Key: (*) greater than value in the absence of LTB_4 ($P < 0.05$), and (†) different from value in the presence of LTB_4 alone ($P < 0.05$).

LTB_4 induced time- and concentration-dependent increases in PA and DG accumulation. Consistent with results using fMLP [32], DG generated in response to LTB_4 was most likely derived from the sequential actions of PLD and phosphatidate phosphohydrolase. Several lines of evidence support this conclusion. First, in the presence of ethanol, LTB_4-induced PEt formation was accompanied by a decrease in PA and DG accumulation, suggesting that both PA and PEt are produced via a common biochemical pathway, i.e. PLD. Second, DG accumulation occurred concomitantly with, or lagged slightly behind, PA accumulation. This is consistent with the proposal that DG was derived from PA. Third, the phosphatidate phosphohydrolase inhibitor propranolol nearly abolished DG generation and markedly potentiated PA accumulation, suggesting that most of the DG accumulation produced in the presence of LTB_4 was derived from the hydrolysis of PA. Finally, in a preliminary experiment, the DG kinase inhibitor, R55949 (3 μM) [37], did not alter LTB_4-induced PA and DG accumulation, suggesting that the PA generated in response to LTB_4 was not derived from a product of PLC activity.

Regarding the mechanism(s) by which LTB_4 activates PLD in neutrophils, we demonstrated that LTB_4-induced PEt accumulation and MPO release were inhibited by the selective LTB_4 antagonist Ly 223982. Furthermore, treatment of human neutrophils with Ly 223982 antagonized LTB_4-induced PA and DG accumulation as well as Ca^{2+} mobilization (unpublished observations). These data

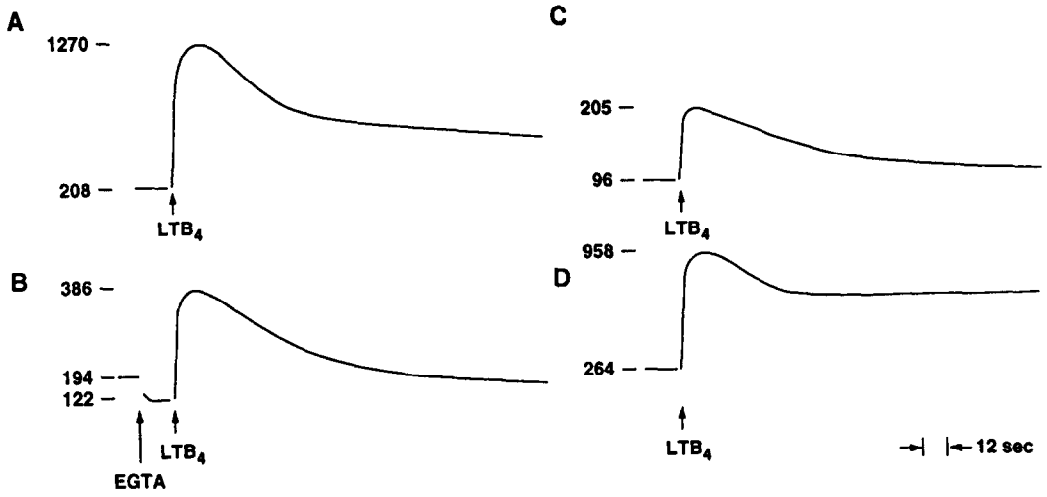

Fig. 8. Role of Ca^{2+} in LTB_4-induced Ca^{2+} mobilization in human neutrophils. Human neutrophils were loaded with fura-2 and treated with 1 μM LTB_4 to monitor Ca^{2+}/fura-2 fluorescence in the presence of (A) 1 mM extracellular Ca^{2+}, (B) 200 μM EGTA added to Ca^{2+}-free buffer 10 sec before LTB_4, (C) 50 μM EGTA in Ca^{2+}-free buffer for 30 min at 37° and (D) same incubation as (C) followed by re-addition of 1 mM Ca^{2+} for 10 min. The arrows indicate the time at which LTB_4 was added. The calculated maximal LTB_4-induced increase in $[Ca^{2+}]_i$ over baseline $[Ca^{2+}]_i$ was (A) 1062 nm, (B) 264 nm, (C) 109 nM and (D) 694 nM. Data presented are representative Ca^{2+} transients induced by LTB_4. Each condition was repeated four times with different donors. The relative change in LTB_4-induced $[Ca^{2+}]_i$ versus the baseline value varied less than 5% among different donors for each of the conditions.

indicate that all LTB_4-induced effects measured were mediated via LTB_4-receptor activation.

To define the post-receptor mechanism(s) by which LTB_4 activates PLD in neutrophils, the roles of Ca^{2+} and PKC were examined. Chelation of extracellular Ca^{2+} substantially reduced, but did not abolish, LTB_4-induced Ca^{2+} mobilization and PLD activation. On the other hand, depleting both intracellular and extracellular Ca^{2+} completely inhibited LTB_4-induced PLD activation and most of the Ca^{2+} mobilization. These results, coupled with the ability of the Ca^{2+} ionophore A23187 to stimulate PLD [32], strongly suggest that agonist-induced PLD activation in human neutrophils is Ca^{2+} dependent. Caution should be exercised in applying these results universally since the role of Ca^{2+} in the activation of PLD in other cell types may be stimulus-specific. For example, whereas thrombin-induced PEt generation in human erythroleukemia cells is Ca^{2+} dependent, PEt formation in response to PMA is Ca^{2+} independent [47]. As a result, it is likely that distinct mechanisms are responsible for PLD activation in response to receptor agonists versus phorbol esters.

That PLD can be activated by multiple mechanisms is highlighted when the role of PKC in the activation of PLD is examined. Like Ca^{2+}, PKC has a multifunctional role in regulating cellular activity. Consistent with this, evidence from various laboratories strongly supports the concept that PLD activation can be mediated by PKC. For example, phorbol esters appear to be universally effective in activating PLD [42], and PKC over-expression enhances phorbol ester-stimulated PLD activity as well as up-regulates thrombin-stimulated PLD activity [48, 49]. In addition, down-regulating PKC by chronic exposure of cells to phorbol esters blocks carbachol-induced PLD activation in astrocytoma cells [50]. Similarly, Natarajan and Garcia [51] reported that down-regulating PKC by prolonged pretreatment of bovine pulmonary artery endothelial cells with phorbol ester abolishes bradykinin-induced PEt formation. Finally, PLD in membranes prepared from Chinese hamster lung fibroblasts can be activated by the addition of purified PKC [52].

Despite the substantial evidence linking a direct stimulation of PKC with activation of PLD, results from other studies on the involvement of PKC in mediating receptor-stimulated activation of PLD have been equivocal. For example, PKC inhibitors fail to reduce agonist-induced PLD activation in ovarian granulosa cells or human neutrophils [22, 53]. In both human neutrophils [54] and rabbit peritoneal neutrophils [55], staurosporine strongly inhibits PMA-induced PEt formation, but, almost paradoxically, stimulates the formation of PEt in response to fMLP. Similar results were generated in the present experiments. That is, staurosporine inhibited PLD activation induced by the PKC activator, PMA, but potentiated LTB_4-induced PLD activation. As the specificity of staurosporine is questionable, other PKC inhibitors with greater selectivity were also tested. The overall effect of these compounds, calphostin C and chelerythrine, was identical to that observed with staurosporine. The mechanism by which PKC inhibitors potentiate LTB_4-induced activation of PLD in human neutrophils is not clear. However, the ability of staurosporine to enhance LTB_4-induced Ca^{2+} mobilization [56], perhaps by reversing a PKC-mediated inhibition of phosphatidylinositol bisphosphate-specific PLC activity [42], offers one potential explanation. Regardless, the inability of staurosporine and other PKC inhibitors to reduce PEt accumulation in these

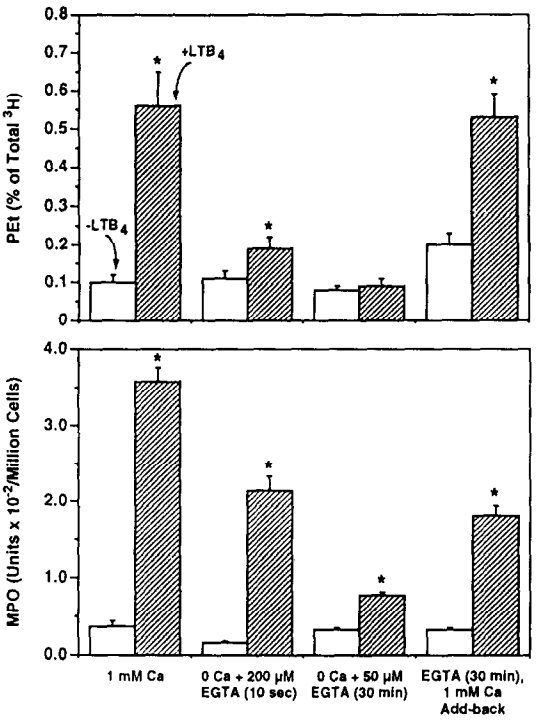

Fig. 9. Role of Ca^{2+} in LTB_4-induced PLD activation and MPO release in human neutrophils. Measurements were made under the following conditions: (1) 1 mM Ca^{2+} KRH; (2) Ca^{2+}-free KRH containing 200 μM EGTA for 10 sec before LTB_4 challenge to remove extracellular Ca^{2+}; (3) Ca^{2+}-free KRH containing 50 μM EGTA for 30 min before LTB_4 challenge to remove extracellular Ca^{2+} and deplete intracellular Ca^{2+}; (4) readdition of Ca^{2+} to the KRH for 10 min after treating cells with 50 μM EGTA for 30 min. PEt (panel A) and MPO release (panel B) were measured at 5 and 3 min after exposure to 1 μM LTB_4, respectively. The open bars represent values in the absence of LTB_4. Hatched bars represent LTB_4-stimulated values. Data are the means ± SEM of 4–5 experiments. Key: (*) significantly greater than basal value ($P < 0.05$).

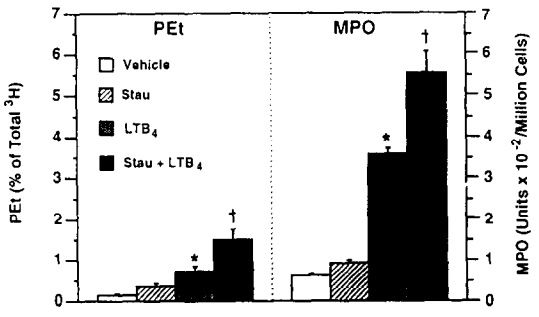

Fig. 10. Effect of staurosporine on LTB_4-induced PLD activation and MPO release in human neutrophils. Cells were exposed to vehicle or 1 μM staurosporine for 5 min before being challenged with 1 μM LTB_4. PEt and MPO release were measured at 5 and 3 min, respectively, after exposure to LTB_4. Data are the means ± SEM of 5 experiments. Key: (*) significantly greater than vehicle value ($P < 0.05$), and (†) significantly greater than value in the presence of LTB_4 alone ($P < 0.05$).

Table 1. Effects of chelerythrine and calphostin C on LTB_4-induced PLD activation

Treatment	PEt (% of total ^{3}H)
Control	0.14 ± 0.02
LTB_4	0.73 ± 0.06*
LTB_4 + chelerythrine	1.19 ± 0.13†
Control	0.19
LTB_4	0.57
LTB_4 + calphostin C	0.75

Cells were incubated with 0.5% ethanol and exposed to vehicle, 5 μM chereythrine or 1 μM calphostin C for 5 min before being exposed to 1 μM LTB_4. PEt accumulation was measured 3 min after LTB_4 treatment. Values are the means ± SEM of 4 experiments (chelerythrine) or data from a single experiment (calphostin C).

* Significantly greater than control value ($P < 0.05$).

† Significantly greater than value in the presence of LTB_4 alone ($P < 0.05$).

experiments suggests that activation of PKC is not critical for the expression of LTB_4-induced PLD activity in human neutrophils.

In terms of the functional role of PLD in the human neutrophil, the present experiments provide several lines of evidence suggesting that LTB_4-induced PLD activation is linked to degranulation, but not to Ca^{2+} mobilization. First, the concentration–response curve for LTB_4-induced PLD activation was superimposable with that for LTB_4-induced degranulation. In contrast, neither the concentration–response curve nor the time-course for LTB_4-induced PLD activation coincided with those for LTB_4-induced Ca^{2+} mobilization. Second, ethanol produced a concentration-dependent inhibition of both PLD activity and degranulation, but had no effect on Ca^{2+} mobilization. Thus, in contrast to results of previous studies suggesting that PA has a role in Ca^{2+} mobilization [11–14], our results failed to provide evidence supporting a role for PLD-derived PA in regulating LTB_4-induced Ca^{2+} influx or release in the human neutrophil. Finally, in an effort to determine which PLD product, PA or DG, was involved in agonist-induced degranulation, experiments were conducted with the phosphatidate phosphohydrolase inhibitor propranolol. In these studies, propranolol abolished DG formation in response to a maximal concentration of LTB_4 but had no effect on degranulation. These results, when viewed in isolation, suggest that if PLD is indeed involved in agonist-stimulated MPO release from human neutrophils, then PA is likely to be a more important messenger than DG. However, propranolol also failed to potentiate LTB_4-induced degranulation, an effect that would be predicted if PA is directly and solely responsible for LTB_4-induced degranulation. The basis for the failure of propranolol to potentiate the functional response to LTB_4 is unclear. One possibility is that PA has a permissive role in the degranulation process, but that other intracellular signals are responsible for the absolute amount of MPO released. Of course, another obvious explanation is that neither DG nor PA is involved in LTB_4-induced degranulation of the human neutrophil. Our data do not discriminate between these possibilities.

In summary, LTB_4 was shown to stimulate PLD activity in human neutrophils. This stimulation was Ca^{2+} dependent but did not appear to require the activation of PKC. In addition, PLD activation was linked to LTB_4-induced neutrophil degranulation, but not Ca^{2+} mobilization. Thus, the results of these experiments support the growing body of evidence that implicates PLD as an important regulator of signal transduction in inflammatory cells.

Acknowledgements—The technical contributions of D. B. Schmidt are greatly appreciated as is the help of D. Lavan in preparing this manuscript.

REFERENCES

1. Bray MS, Leukotrienes in inflammation. *Agents Actions* **19**: 87–99, 1986.
2. Verghese MW, Charles L, Jakoi L, Dillon SB and Snyderman R, Role of a guanine nucleotide regulatory protein in the activation of phospholipase C by different chemoattractants. *J Immunol* **138**: 4374–4380, 1987.
3. Omann GM, Traynor AE, Harris AL and Sklar LA, LTB_4-induced activation signals and responses in neutrophils are short-lived compared to formylpeptide. *J Immunol* **138**: 2626–2632, 1987.
4. Dillon SB, Murray JJ, Verghese MW and Snyderman R, Regulation of inositol phosphate metabolism in chemoattractant-stimulated human polymorphonuclear leukocytes. Definition of distinct dephosphorylation pathways for IP_3 isomers. *J Biol Chem* **262**: 11546–11552, 1987.
5. O'Flaherty JT and Nishihira J, Arachidonate metabolites, platelet-activating factor, and the mobilization of protein kinase C in human polymorphonuclear neutrophils. *J Immunol* **138**: 1889–1895, 1987.
6. Korchak HM, Vosshall LB, Haines KA, Wilkenfeld C, Lundquist KF and Weissmann G, Activation of the human neutrophil by calcium-mobilizing ligands. II. Correlation of calcium, diacyl glycerol, and phosphatidic acid generation with superoxide anion generation. *J Biol Chem* **263**: 11098–11105, 1988.
7. Goldman DW, Gifford LA, Olson DM and Goetzl EJ, Transduction by leukotriene B_4 receptors of increase in cytosolic calcium in human polymorphonuclear leukocytes. *J Immunol* **135**: 525–530, 1985.
8. Sarau HM and Mong S, Co-expression of leuktoriene B_4 and leukotriene D_4 receptors on human monocytic leukemia U-937 cells. *Adv Prostaglandin Thromboxane Leukot Res* **19**: 180–186, 1989.
9. Kanaho Y, Kanoh H, Saitoh K and Nozawa Y, Phospholipase D activation by platelet-activating factor, leukotriene B_4, and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. *J Immunol* **146**: 3536–3541, 1991.
10. Agwu DE, McCall CE and McPhail LC, Regulation of phospholipase D-induced hydrolysis of choline-containing phosphoglycerides by cyclic AMP in human neutrophils. *J Immunol* **146**: 3895–3903, 1991.
11. Salmon DM and Honeyman TW, Proposed mechanism of cholinergic action in smooth muscle. *Nature* **284**: 344–345, 1980.
12. Putney JW Jr, Weiss SJ, Van de Welle CM and Haddas RR, Is phosphatidic acid a calcium ionophore under neurohumoral control. *Nature* **284**: 345–347, 1980.
13. Serhan C, Anderson P, Goodman E, Dunhem P and Weissmann G, Phosphatidate and oxidized fatty acids are calcium ionophores. *J Biol Chem* **256**:2736–2741, 1981.
14. Ohsaki S and Deguchi T, Stimulation by phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. *J Biol Chem* **256**: 10945–10948, 1981.
15. Moolenaar WH, Kruijer W, Tilly BC, Verlaan I, Bierman AJ and de Laat SE, Growth factor-like action of phosphatidic acid. *Nature* **323**: 171–173, 1986.
16. Murayama T and Ui M, Receptor-mediated inhibition of adenylate cyclase and stimulation of arachidonic acid release in 3T3 fibroblasts. *J Biol Chem* **260**: 7226–7233, 1985.
17. Clark RB, Salmon D and Honeyman TW, Phosphatidic acid inhibition of PGE_1-stimulated cAMP accumulation in WI-38 fibroblasts: Similarities with carbachol inhibition. *J Cyclic Nucleotide Res* **6**: 37–49, 1980.
18. Proll MA, Clark RB and Butcher RW, Phosphatidate and monooleylphosphatidate inhibition of fibroblast adenylate cyclase is mediated by the inhibitory coupling protein, N_i. *Mol Pharmacol* **28**: 331–337, 1985.
19. Jackowski S and Rock CO, Stimulation of phosphatidylinositol 4,5-bisphosphate phospholipase C activity by phosphatidic acid. *Arch Biochem Biophys* **268**: 516–524, 1989.
20. Yu C-L, Tsai M-H and Stacey DW, Cellular *ras* activity and phospholipid metabolism. *Cell* **52**: 63–71, 1988.
21. Cockroft, S, Ca^{2+}-dependent conversion of phosphatidylinositol to phosphatidate in neutrophils stimulated with fMet-Leu-Phe or ionophore A23187. *Biochim Biophys Acta* **795**: 37–46, 1984.
22. Liscovitch M and Amsterdam A, Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. Possible role in signal transduction. *J Biol Chem* **264**: 11762–11767, 1989.
23. Simmonds AC and Halsey MJ, General and local anaesthetics perturb the fusion of phospholipid vesicles. *Biochim Biophys Acta* **813**: 331–337, 1985.
24. Rossi F, Grzeskowiak M, Della Bianca V, Calzetti F and Gandini G, Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of the NADPH oxidase by FMLP in human neutrophils. *Biochem Biophys Res Commun* **168**: 320–327, 1990.
25. Agwu DW, McPhail LC, Sozzani S, Bass DA and McCall CE, Phosphatidic acid is a second messenger in human polymorphonuclear leukocytes. Effects on activation of NADPH oxidase. *J Clin Invest* **88**: 531–539, 1991.
26. Bonser RW, Thompson NT, Randall RW and Garland LG, Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem J* **264**: 617–620, 1989.
27. Fällman M, Lew DP, Stendahl O and Andersson T, Receptor-mediated phagocytosis in human neutrophils is associated with increased formation of inositol phosphates and diacylglycerol. *J Clin Invest* **84**: 886–891, 1989.
28. Lin P, Wiggan GA and Gilfillan AM, Activation of phospholipase D in a rat mast (RBL 2H3) cell line. A possible unifying mechanism for IgE-dependent degranulation and arachidonic acid metabolite release. *J Immunol* **146**: 1609–1616, 1981.
29. Lin P, Wiggan GA, Welton AI and Gilfillan AM, Differential effects of propranolol on the IgE-dependent, or calcium ionophore-stimulated, phosphoinositide hydrolysis and calcium mobilization in a mast (RBL 2H3) cell line. *Biochem Pharmacol* **41**: 1941–1948, 1991.
30. Bauldry SA, Bass DA, Cousart SL and McCall CE, Tumor necrosis factor α priming of phospholipase D in human neutrophils. Correlation between phosphatidic acid production and superoxide generation. *J Biol Chem* **266**: 4173–4179, 1991.
31. Böyum A, Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest* **21** (Suppl 97): 77–89, 1968.
32. Chabot MC, McPhail LC, Wykle RL, Kennerly DA

and McCall CE, Comparison of diglyceride production from choline-containing phosphoglycerides in human neutrophils stimulated with fMLP, A23187, or PMA. *Biochem J* **286**: 693–699, 1992.
33. Bligh EG and Dyer W, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911–917, 1959.
34. Billah MM, Pai JK, Mullmann TJ, Egan RW and Siegel MI, Regulation of phospholipase D in HL-60 granulocytes. Activation by phorbol esters, diglyceride and calcium ionophore via protein kinase-independent mechanisms. *J Biol Chem* **264**: 9069–9076, 1989.
35. Winkler JM, Sarau HM, Foley JJ, Mong S and Crooke ST, Leukotriene B_4-induced homologous desensitization of calcium mobilization and phosphoinositide metabolism in U-937 cells. *J Pharmacol Exp Ther* **246**: 204–210, 1988.
36. Bradley PP, Priebat DA, Christensen RD and Rothstern G, Measurement of cutaneous inflammation: Estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78**: 206–209, 1982.
37. Courcelles D de C, The use of diacylglycerol kinase inhibitors for elucidating the roles of protein kinase C. In: *The Biology and Medicine of Signal Transduction* (Ed. Nishizuka Y), pp. 491–496. Raven Press, New York, 1990.
38. Gapinski DM, Mallett BE, Froelich LL and Jackson WT, Benzophenone dicarboxylic acid antagonists of leukotriene B_4. 2. Structure–activity relationships of the lipophilic side chain. *J Med Chem* **33**: 2807–2813, 1990.
39. Anthes JC, Krasovsky J, Egan RW, Siegel MI and Billah MM, Sequential degradation of choline phosphoglycerides by phospholipase D and phosphatidate phosphohydrolase in dibutyryl cAMP-differentiated U937 cells. *Arch Biochem Biophys* **287**: 53–59, 1991.
40. Mullmann TJ, Siegel MI, Egan RW and Billah MM, Complement C_{5a} activation of phospholipase D in human neutrophils. A major route to the production of phosphatidates and diglycerides. *J Immunol* **144**: 1901–1908, 1990.
41. Thompson NT, Bonser RW and Garland LG, Receptor-coupled phospholipase D and its inhibition. *Trends Pharmacol Sci* **12**: 404–408, 1991.
42. Shukla SD and Halenda SP, Phospholipase D in cell signalling and its relationship to phospholipase C. *Life Sci* **48**: 851–866, 1991.
43. Herbert JM, Augereau JM, Gleye J and Maffrand JP, Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **172**: 983–999, 1990.
44. Kobayashi E, Nakaro H, Morimoto M and Tamaoki T, Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* **159**: 548–553, 1989.
45. Tamoki T, Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods Enzymol* **201**: 340–347, 1991.
46. Uings IJ, Thompson NT, Randall NW, Spacey GD, Bonser RW, Hudson AT and Garland LG, Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophils. *Biochem J* **281**: 597–600, 1992.
47. Halenda SP and Rehm AG, Evidence for the calcium-dependent activation of phospholipase D in thrombin-stimulated human erythroleukemia cells. *Biochem J* **267**: 479–483, 1990.
48. Pai J-K, Pachter JA, Weinstein IB and Bishop WR, Overexpression of protein kinase C β1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proc Natl Acad Sci USA* **88**: 598–602, 1991.
49. Pachter JA, Pai J-K, Mayer-Ezell R, Petrin JM, Dobek E and Bishop WR, Differential regulation of phosphoinositide and phosphatidylcholine hydrolysis by protein kinase C-β1 overexpression. *J Biol Chem* **267**: 9826–9830, 1992.
50. Martinson EA, Trilivas I and Brown JH, Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J Biol Chem* **265**: 22282–22287, 1990.
51. Natarajan V and Garcia JGN, Bradykinin, phorbol esters and aluminium fluoride induce phospholipase D activation in endothelial cells. *FASEB J* **4**: A1781, 1990.
52. Conricode KM, Brewer KA and Exton JM, Activation of phospholipase D by protein kinase. *J Biol Chem* **267**: 7199–7202, 1992.
53. Mullmann TJ, Siegel MI, Egan RW and Billah MM, Sphingosine inhibits phosphatidate phosphohydrolase in human neutrophils by a protein kinase C-independent mechanism. *J Biol Chem* **266**: 2013–2016, 1991.
54. Reinhold S, Prescott SM, Zimmerman GA and McIntyre TM, Activation of human neutrophil phospholipase C by three separable mechanisms. *FASEB J* **4**: 208–214, 1990.
55. Kanaho Y, Nishida A and Nozawa Y, Calcium rather than protein kinase C is the major factor to activate phospholipase D in FMLP-stimulated rabbit peritoneal neutrophils. *J Immunol* **149**: 622–628, 1992.
56. Wong K, Kwan-Yeung L and Turkson J, Staurosporine clamps cytosolic free Ca^{2+} concentrations of human neutrophils. *Biochem J* **283**: 499–505, 1992.