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# Effect of Wortmannin and 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) on *N*-Formyl-Methionyl-Leucyl-Phenylalanine-Induced Phospholipase D Activation in Differentiated HL60 Cells

## POSSIBLE INVOLVEMENT OF PHOSPHATIDYLINOSITOL 3-KINASE IN PHOSPHOLIPASE D ACTIVATION

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**ABSTRACT.** Phospholipase D (PLD) plays an important role in neutrophil activation. However, despite various proposed mechanisms, its detailed regulatory mechanism is not fully understood. The functional coupling between phosphatidylinositol 3-kinase (PI-3 kinase) and PLD was investigated in *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated human promyelocytic leukemia HL60 cells, using wortmannin, a fungal metabolite that is known as a selective inhibitor for phosphatidylinositol 3-kinase. Treatment of cells with this drug inhibited the formation of both phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$), a product of PI 3-kinase, and phosphatidylbutanol (PBut), the specific product of transphosphatidylation due to PLD in the presence of butanol, with similar concentration dependence ($IC_{50}$ = 30–70 nM). Another PI 3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) also inhibited PBut formation in a concentration-dependent manner. However, wortmannin failed to inhibit phorbol 12-myristate 13-acetate-induced PLD activation in whole cells and membrane PLD activity in an *in vitro* assay system, indicating that inhibition of fMLP-induced PLD activation by wortmannin was not due to its direct effect on PLD activity. These results suggest that a major part of inhibition of PLD activation by wortmannin might be mediated through its effect on PI 3-kinase. BIOCHEM PHARMACOL **53**;12:1929–1936, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** phospholipase D; phosphatidylinositol 3-kinase; wortmannin; LY294002; HL60 cell

Activation of cell surface receptors results in the formation of second messengers, which transduce their signal to the cell interior. Several kinds of intracellular messengers are produced from membrane phospholipids by the action of signal-transducing phospholipases [1–3]. In addition to phospholipases C and $A_2$, receptor-mediated activation of PLD§ has been shown recently to play a pivotal role in signal transduction [4, 5]. In neutrophils, correlation between PLD activation and secretory response [6, 7] or superoxide generation [8] has been suggested. Therefore, PLD may play an important part in neutrophil activation. Several factors have been proposed for PLD activation [4, 5], such as $Ca^{2+}$, PKC, protein tyrosine kinase, and GTP-binding proteins. However, details of its regulatory mechanism are not fully understood.

PI 3-kinase catalyzes phosphorylation at the D-3 position of phosphoinositides [9, 10]. PI 3-kinase is activated upon stimulation of growth factor receptor, cytokine receptor, insulin receptor, or GTP-binding protein-coupled receptor. Activation of the fMLP receptor in neutrophils, a GTP-binding protein-coupled receptor, results in the accumulation of $PIP_3$ [11–13]. Although the exact functions of PI 3-kinase (or its product $PIP_3$) are unknown, a possible implication has been demonstrated in several signal transduction events, such as activation of Ras [14] and Rac [15], activation of PKCζ [16, 17], and actin polymerization [11, 18].

A fungal metabolite, wortmannin, is known to be a potent anti-inflammatory agent and inhibits agonist-in-

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§ *Abbreviations:* PLD, phospholipase D; PI 3-kinase, phosphatidylinositol 3-kinase; $PIP_3$, phosphatidylinositol 3,4,5-trisphosphate; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PKC, protein kinase C; GTP-binding proteins, guanine nucleotide-binding proteins; PBut, phosphatidylbutanol; PA, phosphatidic acid; 1,2-DG, 1,2-diacylglycerol; PC, phosphatidylcholine; AA, arachidonic acid; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; $LTB_4$, leukotriene $B_4$; PMA, phorbol 12-myristate 13-acetate; and GTPγS, guanosine 5′-3-O-(thio)triphosphate.

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duced neutrophil superoxide generation [12, 13, 19] and actin polymerization [12]. Wortmannin has often been used as a selective inhibitor for PI 3-kinase [20, 21]. The present study was undertaken to examine the functional coupling between PI 3-kinase and PLD, using wortmannin. It was shown that the drug inhibited both PI 3-kinase and PLD in similar concentration-dependent manners in fMLP-activated human promyelocytic leukemia HL60 cells. Furthermore, another PI 3-kinase inhibitor, LY294002 [22, 23], also inhibited PLD activation. In contrast to the receptor-mediated PLD activation, wortmannin failed to inhibit the PLD activation induced in intact cells by the receptor-bypassing agonist PMA and to inhibit membrane PLD activity in *in vitro* assay. These results suggest that inhibition of PLD activation by wortmannin might be mediated through its effect on PI 3-kinase.

## MATERIALS AND METHODS

### *Materials*

Fetal bovine serum was purchased from Irvine Scientific (Santa Ana, CA, U.S.A.). RPMI 1640 medium was from Gibco BRL (Grand Island, NY, U.S.A.). [9,10-$^3$H]Oleic acid (9.2 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA, U.S.A.). [5,6,8,9,11,12,14,15-$^3$H]AA (214 Ci/mmol) and phosphorus-32 (2 mCi/mL) were from Amersham International (Aylesbury, U.K.). fMLP, PMA, A23187, dibutyryl cyclic 3′,5′-AMP, cytochalasin B, BSA, and wortmannin were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). LY294002 was from the Alexis Corp. (San Diego, CA, U.S.A.). $LTB_4$ was a gift from the Ono Pharmaceutical Co. (Osaka, Japan). GTPγS was obtained from Boehringer Mannheim (Mannheim, Germany). PBut standard was produced from egg PC by using crude cabbage PLD by the method of Yang *et al.* [24]. Silica gel LK6D and silica gel 60 plates were purchased from Whatman (Clifton, NJ, U.S.A.) and Merck (Darmstadt, Germany), respectively. X-Omat AR films were from Kodak (Rochester, NY, U.S.A.). The HL60 cell line was supplied by Dr. T. Okazaki (Osaka Dental University, Japan). Other reagents were of analytical grade.

### *Cell Culture*

HL60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37°. For differentiation, cells were transferred to serum-free RPMI 1640 medium supplemented with 5 μg/mL transferrin and 5 μg/mL insulin, and cultured for 24 hr. HL60 cells were then differentiated toward neutrophil-like cells with 0.5 mM dibutyryl cyclic AMP for 72 hr.

### *[$^3$H]PBut Formation in Intact HL60 Cells*

For PLD assay, cells were labeled with [$^3$H]oleic acid (0.5 μCi/mL) for the last 12–15 hr [25]. [$^3$H]Oleic acid-labeled cells were washed twice with buffer A, consisting of 136 mM NaCl, 4.9 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES (pH 7.4). Lipids were extracted according to the method of Bligh and Dyer [26]. About 55% of total lipid radioactivity was found in the PC fraction (data not shown). The cells ($0.8 \times 10^6$ cells/500 μL) were preincubated in buffer A containing 1 mM $CaCl_2$ at 37° with the indicated concentrations of wortmannin or LY294002 for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated with 0.1 μM fMLP, 1 μM $LTB_4$, or 100 nM PMA in the presence of 0.3% butanol. The reaction was terminated at the indicated times by the addition of 2 mL chloroform:methanol (1:2, v/v) and then lipids were extracted. Authentic PBut was added to each sample as an internal standard, and phospholipids were separated by silica gel LK6D plates. The plates were developed with an upper phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (13:2:3:10, by vol.) [27]. Spots were visualized by iodine vapor, the area corresponding to [$^3$H]PBut was scraped off the plate, and radioactivity was measured in a liquid scintillation counter (Beckman LS 6500). The amount of [$^3$H]PBut is expressed as the percentage of the total radioactivity recovered from the TLC plates.

### *AA Release in Intact HL60 Cells*

AA release by HL60 cells was assayed as previously described [28]. Cells were suspended at $3.5 \times 10^6$ cells/mL in RPMI 1640 medium supplemented with 5 μg/mL transferrin, 5 μg/mL insulin, and 3 mg/mL BSA, and labeled with [$^3$H]AA (1 μCi/mL) at 37° for 1.5 hr. Cells were washed twice with buffer A. Cells ($1.4 \times 10^6$ cells/250 μL) were preincubated in buffer A containing 1 mM $CaCl_2$ at 37° with or without wortmannin for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated with 0.1 μM fMLP in the presence of 3 mg/mL BSA. The reaction was terminated at the indicated times by the addition of 12.5 μL of 0.4 M formic acid and 1 mL of ethyl acetate, and lipids were extracted two times with 1 mL of ethyl acetate. About 90% of total lipid radioactivity was found in the phospholipid fraction (data not shown). Authentic AA was added to each sample as an internal standard, and phospholipids were separated by silica gel LK6D plates. The plates were developed with an upper phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (90:50:20:100, by vol.) [29]. Spots were visualized by iodine vapor, the area corresponding to [$^3$H]AA was scraped off the plate, and radioactivity was measured in a liquid scintillation counter (Beckman LS 6500).

### *[$^3$H]PBut Formation in HL60 Membranes*

PLD activity in HL60 membrane fraction was assayed as described previously [25]. The cells labeled with [$^3$H]oleic acid (0.5 μCi/mL) were washed twice with buffer B, consisting of 0.5 mM ATP, 1 mM EGTA, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 25 mM HEPES (pH 7.4). Cells were then disrupted by nitrogen cavitation (600 p.s.i. for 30 min), and the lysate was centrifuged for 5 min at 500 *g* to remove unbroken cells and nuclei. The resulting supernatant was further centrifuged at 105,000 *g* for 60 min at 4°. The membrane pellet was washed once and finally suspended in buffer B. Isolated HL60 membranes (40 μg protein/assay) and HL60 cytosol (40 μg protein/assay) were suspended in buffer B (100 μL) containing $CaCl_2$ to give a final free $Ca^{2+}$ concentration of 1 μM and were incubated with 100 nM PMA, 10 μM GTPγS, or both 100 nM PMA and 10 μM GTPγS at 37° for 15 min in the presence of 0.3% butanol. The reaction was terminated by the addition of 0.8 mL chloroform: methanol (1:2, v/v). Lipid extraction and analysis were performed as described above.

### *PI 3-Kinase Activity Assay*

To monitor PI 3-kinase activity, $PIP_3$ production was measured according to the method described by Okada *et al.* [13]. Differentiated HL60 cells were suspended at $10^7$ cells/mL in phosphate-free RPMI 1640 medium. After incubation at 37° for 15 min with $^{32}P_i$ (500 μCi/mL), the cell suspension (100 μL) was preincubated at 37° with or without wortmannin for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated for 30 s with 0.1 μM fMLP. Reaction was terminated by 0.6 mL chloroform:methanol:HCl (20:40:1, by vol.). Lipid extraction was performed as described above. The extracted lipids were separated on silica gel 60 plates, impregnated with 1.2% potassium oxalate, which were preactivated at 110° for 20 min just before use. The plates were developed with chloroform:acetone:methanol:acetic acid:water (80:30:26: 24:14, by vol.) [13]. Labeled phospholipids were detected by autoradiography on X-Omat AR films. The area corresponding to [$^{32}$P]$PIP_3$ was scraped off the plate, and radioactivity was determined in a liquid scintillation counter (Beckman LS 6500). The amount of [$^{32}$P]$PIP_3$ is expressed as a percentage of the total radioactivity recovered from the TLC plates.

### *Statistical Analyses*

Data are expressed as means ± SD. The statistical evaluation of the data was performed by one-way analysis of variance followed by the Bonfferoni modified *t*-test. The $IC_{50}$ values were determined graphically or by computerized regression analysis of log concentrations versus enzyme activity. The correlation between parameters was assessed by linear regression analysis.

**TABLE 1. Effect of wortmannin on PMA-induced [$^3$H]PBut formation in undifferentiated and differentiated HL60 cells**

| Treatment | [$^3$H]PBut formation (% of total) | |
|---|---|---|
| | Undifferentiated cells | Differentiated cells |
| None | 0.04 ± 0.00 | 0.12 ± 0.01 |
| fMLP (100 nM) | 0.05 ± 0.01 | 1.41 ± 0.34 |
| PMA (100 nM) | 0.37 ± 0.01 | 0.52 ± 0.13 |
| PMA + 100 nM wortmannin | | 0.49 ± 0.04 |
| PMA + 1 μM wortmannin | 0.46 ± 0.00 | 0.49 ± 0.08 |

HL60 cells labeled with [$^3$H]oleic acid for 12 hr were preincubated with indicated concentrations of wortmannin for 10 min and 5 μM cytochalacin B for the last 5 min, and then stimulated with 0.1 μM fMLP or 100 nM PMA for 5 min in the presence of 0.3% butanol. Analysis of [$^3$H]PBut was performed as described in "Materials and Methods". The total radioactivity recovered from the TLC plates was 786,900 ± 2,190 dpm. Data are means ± SD from two different experiments each performed in duplicate.

## RESULTS

### *Inhibition of fMLP-Induced PLD Activation by Wortmannin*

PLD catalyzes the transphosphatidylation reaction in addition to hydrolysis. In the presence of primary alcohols (e.g. ethanol, butanol), metabolically stable phosphatidylalcohol is produced, which is a specific and useful parameter for PLD activity [4, 5]. In the present study, the accumulation of [$^3$H]PBut was measured as the definitive assay for PLD activity in [$^3$H]oleic acid-labeled HL60 cells in the presence of 0.3% butanol. Pretreatment of neutrophils with cytochalasin B greatly potentiates agonist-induced PLD activation [6, 30], although cytochalasin B alone causes very little stimulation. In differentiated HL60 cells, fMLP-induced [$^3$H]PBut formation was augmented by 5- to 6-fold in the presence of cytochalasin B (data not shown). Therefore, the cells were preincubated for 5 min with 5 μM cytochalasin B prior to fMLP stimulation. fMLP failed to activate PLD in undifferentiated HL60 cells (Table 1). However, in HL60 cells differentiated by incubation with 0.5 mM dibutyryl cyclic AMP for 72 hr, fMLP stimulated the production of [$^3$H]PBut, with the maximal level at 0.1 to 1 μM fMLP (data not shown). When differentiated HL60 cells were pretreated with wortmannin for 10 min, fMLP-induced PBut formation was inhibited in a concentration-dependent manner with an $IC_{50}$ value of around 70 nM (Fig. 1). Basal PBut formation was unaffected by the drug (Fig. 1).

### *Inhibition of fMLP-Induced PLD Activation by LY294002*

Recently, LY294002, another selective inhibitor of PI 3-kinase, has been used [22, 23]. To further support our data obtained with wortmannin, the effect of LY29002 on

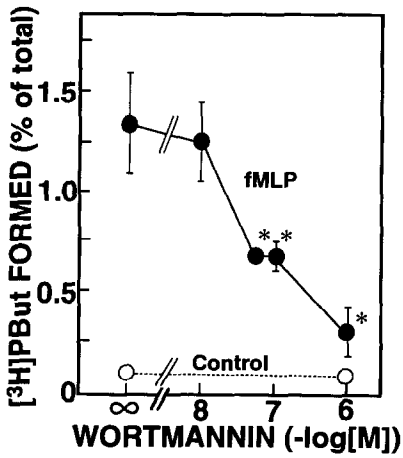


FIG. 1. Concentration-dependent effect of wortmannin on fMLP-induced PBut formation in differentiated HL60 cells. [$^3$H]Oleic acid-labeled differentiated HL60 cells were preincubated with various concentrations of wortmannin for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated for 5 min with 0.1 μM fMLP in the presence of 0.3% butanol. Analysis of [$^3$H]PBut was performed as described in "Materials and Methods". Total radioactivity recovered from the TLC plates was 689,900 ± 98,310 dpm. Data are means ± SD from three different experiments, each performed in duplicate. The unstimulated control value was 0.13 ± 0.10%. Key: (*) significantly different ($P < 0.05$) from fMLP-stimulated sample without wortmannin.

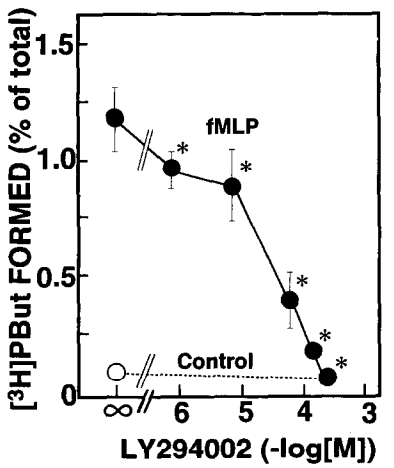


FIG. 2. Concentration-dependent effect of LY294002 on fMLP-induced PBut formation in differentiated HL60 cells. [$^3$H]Oleic acid-labeled differentiated HL60 cells were preincubated with various concentrations of LY294002 for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated for 5 min with 0.1 μM fMLP in the presence of 0.3% butanol. Analysis of [$^3$H]PBut was performed as described in "Materials and Methods". Total radioactivity recovered from TLC plates was 877,100 ± 40,460 dpm. Data shown are means ± SD from three different experiments, each performed in duplicate. The unstimulated control value was 0.17 ± 0.10%. Key: (*) significantly different ($P < 0.05$) from fMLP-stimulated sample without LY294002.

fMLP-induced PBut formation was examined. When differentiated HL60 cells were pretreated with LY294002 for 10 min, fMLP-induced PBut formation was inhibited in a concentration-dependent manner with an $IC_{50}$ value of around 50 μM (Fig. 2). Basal PBut formation was unaffected by the drug (Fig. 2).

### *Effect of Wortmannin on PMA-Induced PLD Activation*

PKC is implicated in agonist-induced activation of PLD [4, 5]. PMA, a PKC activator, is known to activate PLD in various cell types [4, 5], including HL60 cells [7]. As shown in Table 1, PMA (100 nM) stimulated [$^3$H]PBut formation in both undifferentiated and differentiated HL60 cells. Wortmannin at 1 μM, which nearly completely (80%) prevented fMLP-induced PLD activation, had no effect on PMA-induced [$^3$H]PBut accumulation. $Ca^{2+}$ is also known to be a regulatory factor for PLD in a variety of cell types [4, 5]. PLD is activated by fMLP in the presence of $Ca^{2+}$, but not in the absence of extracellular $Ca^{2+}$ (data not shown). A23187, a $Ca^{2+}$ ionophore, has been shown to stimulate PLD in some types of cells, including neutrophils [4, 5, 31]. Therefore, we further examined the effect of wortmannin on A23187-induced [$^3$H]PBut formation. However, A23187 (up to 2 μM) failed to induce [$^3$H]PBut formation in both undifferentiated and differentiated HL60 cells (data not shown).

### *Effects of Wortmannin on fMLP-Induced AA Release and $LTB_4$-Induced PLD Activation*

Cross *et al.* [32] recently have reported that wortmannin inhibits AA release as well as PI 3-kinase activity in bombesin-stimulated Swiss 3T3 cells. $LTB_4$, a major AA metabolite of the lipoxygenase pathway in neutrophils, functions as a potent chemotactic factor that induces adhesion, aggregation, and secretion [33]. $LTB_4$ has been known to induce activation of PLD in neutrophils [6, 34]. Therefore, it is possible that $LTB_4$ synthesized upon stimulation may stimulate and/or amplify PLD activity. The effects of wortmannin on AA release and $LTB_4$-mediated PLD activation were examined. fMLP stimulated the AA release in differentiated HL60 cells, which reached a maximal level at 0.1 to 1 μM fMLP (data not shown). When differentiated HL60 cells were pretreated with wortmannin for 10 min, fMLP-induced AA release was inhibited in a concentration-dependent manner (Fig. 3). However, the $IC_{50}$ value (around 300 nM) was much higher than that for fMLP-mediated PBut formation.

$LTB_4$ stimulated the production of [$^3$H]PBut in differentiated HL60 cells, although the amount was only about 20% of that stimulated by fMLP (data not shown). When HL60 cells were pretreated with various concentrations of wortmannin for 10 min, the drug appeared to produce concentration-dependent inhibition on the $LTB_4$-induced PBut formation (data not shown), but it was not statistically significant.

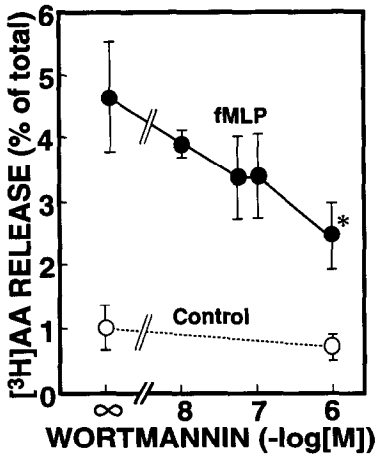


FIG. 3. Effect of wortmannin on fMLP-induced AA release in differentiated HL60 cells. [$^3$H]AA-labeled differentiated HL60 cells were preincubated with various concentrations of wortmannin for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated for 5 min with 0.1 μM fMLP in the presence of 3 mg/mL BSA. Measurement of [$^3$H]AA was performed as described in "Materials and Methods". Total radioactivity recovered from TLC plates was 57,550 ± 4,750 dpm. Data are means ± SD from two different experiments, each performed in duplicate. The unstimulated control value was 1.0 ± 0.4%. Key: (*) significantly different ($P < 0.05$) from fMLP-stimulated sample without wortmannin.

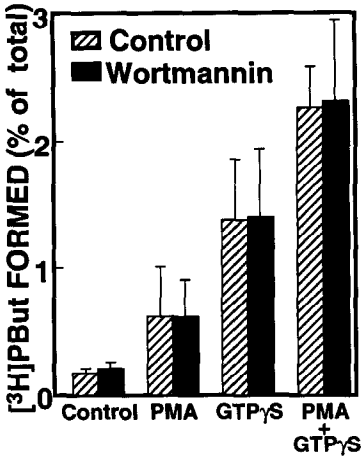


FIG. 4. Effect of wortmannin on PBut formation in HL60 membranes. Isolated HL60 membranes (40 μg protein) and HL60 cytosol (40 μg protein) were incubated with 100 nM PMA, 100 μM GTPγS, or both PMA and GTPγS for 15 min in the absence or presence of 100 nM wortmannin. Measurement of [$^3$H]PBut was performed as described in "Materials and Methods". Total radioactivity recovered from TLC plates was 1,104,000 ± 168,800 dpm. Data are means ± SD from at least two different experiments, each performed in duplicate.

### *Effect of Wortmannin on HL60 Membrane PLD Activity* In Vitro

To study the inhibitory mechanism further, the effect of wortmannin on membrane PLD activity was examined. As reported previously [25], membrane PLD activity is enhanced by PMA and/or GTPγS in the presence of the cytosol fraction from HL60 cells. However, as shown in Fig. 4, wortmannin had no effect on membrane PLD activity in *in vitro* assay systems. Also, the basal PLD activity was not affected by the drug. These results suggest that wortmannin may not exert a direct inhibitory effect on PLD activity.

### *Inhibition by Wortmannin of fMLP-Induced $PIP_3$ Production*

Wortmannin is known to bind selectively to PI 3-kinase and inhibit its activity [20, 21]. PI 3-kinase activity was measured by the formation of [$^{32}$P]$PIP_3$ in $^{32}$P-labeled differentiated HL60 cells. In these experiments, HL60 cells were labeled for 15 min with $^{32}P_i$. Detection of fMLP-stimulated $PIP_3$ formation became less distinct with longer incubation of cells with $^{32}P_i$ (data not shown). Figure 5A shows a typical autoradiogram of a TLC plate of $^{32}$P-labeled phospholipids from $^{32}$P-labeled cells. fMLP stimulated $^{32}$P incorporation into the $PIP_3$ fraction, indicating PI 3-kinase activation. Pretreatment of cells with wortmannin prior to fMLP stimulation prevented [$^{32}$P]$PIP_3$ accumulation (Fig. 5B). Inhibition was concentration dependent with an $IC_{50}$ value of around 30 nM. This inhibitory profile was similar to that for fMLP-induced PBut formation (Fig. 1). Recent reports [13, 23] have demonstrated that wortmannin did not inhibit the fMLP-stimulated incorporation of $^{32}$P into PA in neutrophils. In differentiated HL60 cells, fMLP-induced $^{32}$P labeling of PIP, $PIP_2$, and PA was not inhibited by wortmannin (Fig. 5A). The radioactivities of fMLP-stimulated samples with or without 1 μM wortmannin were 3230 ± 420 and 2600 ± 360 cpm in PIP, 3910 ± 1300 and 3750 ± 1470 cpm in $PIP_2$, and 3740 ± 470 and 3980 ± 450 cpm in PA, respectively. These data suggest that the drug did not affect [$^{32}$P]ATP labeling. As shown in Fig. 6, there was a positive correlation between the levels of $PIP_3$ and PBut in the presence of wortmannin, with the correlation coefficient being 0.90. In our assay system, no increase could be detected in the $PIP_3$ fraction by PMA (100 nM) (data not shown).

## DISCUSSION

PLD has been regarded as an important signal transducing enzyme in a wide variety of cells. PLD hydrolyzes PC to generate PA, which is subsequently metabolized to 1,2-DG via PA phosphohydrolase or to lyso-PA via phospholipase $A_2$ [4]. The possible involvement of $Ca^{2+}$, PKC, protein tyrosine kinase, or GTP-binding proteins has been demonstrated for regulation of its activity. In some cases, PLD activation is dependent on PKC downstream of PLC activation [35, 36]. However, in other cases including fMLP-stimulated neutrophils [30], PKC plays, if any, a minor role. The mechanism(s) of fMLP-mediated PLD activation is not clearly understood.

Wortmannin has been known as an anti-inflammatory drug [37]. The drug inhibits several neutrophil functions [12, 13, 19–21]. Although it has been reported that the

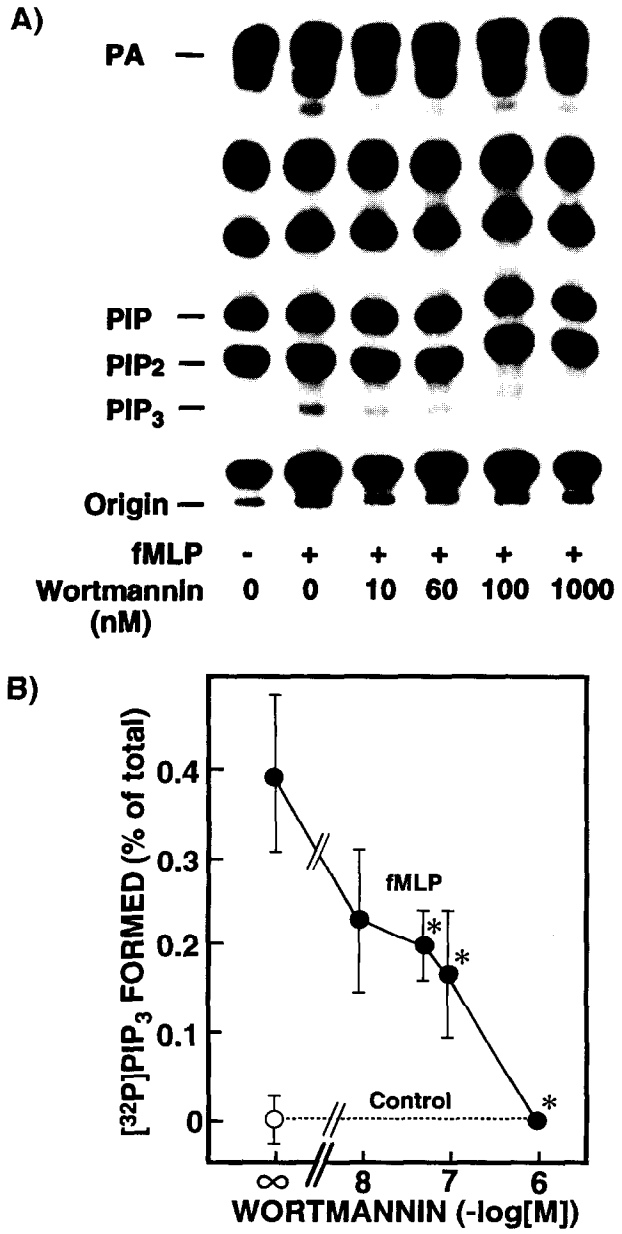


FIG. 5. Effect of wortmannin on fMLP-induced [$^{32}$P]$PIP_3$ formation in differentiated HL60 cells. Differentiated HL60 cells were labeled with $^{32}$P-phosphate for 15 min in phosphate-free RPMI 1640. Cells were preincubated with various concentrations of wortmannin for 10 min and with 5 μM cytochalasin B for the last 5 min, and then stimulated for 30 s with 0.1 μM fMLP. Phospholipids were extracted and separated as described in "Materials and Methods". Labeled phospholipids were detected by autoradiography on Kodak X-Omat AR films. (A) Typical autoradiogram of TLC plate. Lower panel (B) shows the changes of [$^{32}$P]$PIP_3$ expressed as a percentage of the total radioactivity. Total radioactivity recovered from TLC plates was 17,130 ± 2,190 cpm. The radioactivity of $PIP_3$ was counted in a scintillation counter. Data are means ± SD from at least three different experiments. Key: (*) significantly different ($P < 0.05$) from fMLP-stimulated sample without wortmannin.

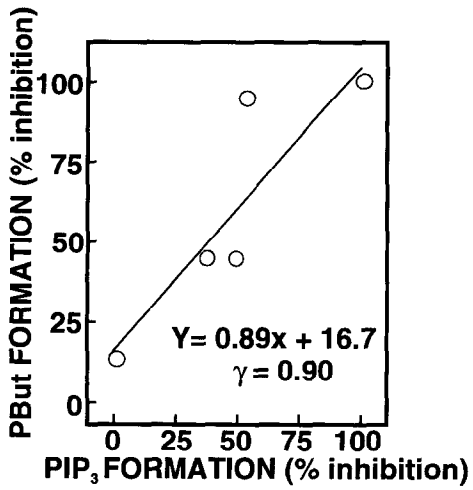


FIG. 6. Relationship between inhibition of [$^3$H]PBut formation and [$^{32}$P]$PIP_3$ formation by wortmannin in differentiated HL60 cells. The results obtained in Figs. 1 and 5B were remodeled. Data are expressed as inhibition percentages of [$^3$H]PBut formation and [$^{32}$P]$PIP_3$ formation by wortmannin by defining values obtained without drug as 100% and values obtained without fMLP as 0%. The correlation coefficient was obtained with linear regression analysis.

drug inhibits PLD activity in neutrophils [38], the exact mechanism is unknown. Recently, wortmannin has become appreciated as a specific inhibitor for PI 3-kinase [20, 21], which binds to the 110 kDa catalytic subunit of the heterodimer enzyme [20]. These findings led us to examine the possible coupling of PI 3-kinase and PLD in fMLP-stimulated HL60 cells. Wortmannin was shown to inhibit fMLP-induced PLD activation (Fig. 1). However, it failed to inhibit PLD activation induced by the receptor-bypassing agonist PMA in HL60 cells. Wortmannin has been reported recently to inhibit bombesin-stimulated AA release in Swiss 3T3 cells with an $IC_{50}$ value around 2 nM [32], which is lower than that observed for fMLP-induced PBut formation in HL60 cells (around 70 nM). Since AA metabolites, especially $LTB_4$, enhance agonist-induced neutrophil responses, it is possible that the blockage of PLD activation by wortmannin is secondary to the inhibition of AA release. However, this is not likely, because the $IC_{50}$ value of wortmannin for fMLP-induced AA release (300 nM) is considerably higher than that for PBut formation (70 nM). Moreover, PBut formation by $LTB_4$ was only one-fifth of that caused by fMLP in differentiated HL60 cells. In an *in vitro* assay system, wortmannin had no effect on membrane PLD activity stimulated by PMA, GTPγS, or both (Fig. 4). These results suggest that wortmannin does not directly inhibit PLD and also has no effect on PLD activation by PKC and/or GTP binding proteins. Thus, it may be possible that the drug interferes with some signal transduction molecule(s), which is located upstream of PKC and/or GTP-binding proteins.

It was reported that 50 nM wortmannin inhibited fMLP-stimulated phosphatidylethanol (PEt) formation by 67% in the presence of 0.5% ethanol in human neutrophils [38]. In

our present study, wortmannin at 50 nM suppressed fMLP-induced PBut formation by 60% (Fig. 1), similar to that in human neutrophils. On the other hand, in rat adipocytes, insulin-induced PEt formation was fully inhibited by 0.1 μM wortmannin [39]. Although the exact mechanism for this discrepancy between neutrophils and adipocytes is not known, it may be due to different cell types and/or experimental conditions. LY294002, another PI 3-kinase inhibitor, also inhibited fMLP-induced PBut formation in HL60 cells ($IC_{50}$ = 50 μM). Higher concentrations of LY294002 than of wortmannin were necessary to inhibit PLD. It has been reported that the concentration of LY294002 (50 μM) required to inhibit PI 3-kinase activity in guinea pig neutrophils is more than 100-fold higher [23].

Wortmannin inhibited fMLP-induced accumulation of $PIP_3$ in HL60 cells with an $IC_{50}$ value around 30 nM (Fig. 5B). This value is almost the same as that observed in fMLP-stimulated $PIP_3$ formation in guinea pig neutrophils [13]. The inhibitory profile of wortmannin for fMLP-induced PBut formation was correlated well with that for $PIP_3$ formation, as shown in Fig. 6. These observations suggest that inhibition of PLD activity by wortmannin could be mediated through its inhibitory effect on PI 3-kinase. Increasing evidence indicates that PI 3-kinase plays a role in the signal transduction system [9, 10]. It is activated following receptor activation by growth factors, cytokines, and various hormones. The PI 3-kinase first cloned and characterized is a heterodimer consisting of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit [40, 41]. Since the latter contains two SH2 domains, association of PI 3-kinase with tyrosine phosphorylated proteins gives rise to activation of the enzyme. Activation of GTP-binding protein-coupled receptors, such as fMLP receptor in neutrophils [11–13], can also induce the accumulation of $PIP_3$. Recently, a novel PI 3-kinase (PI3-Kγ) has been identified which can be activated by the βγ subunit of GTP-binding protein [42, 43]. The activities of both types of enzymes are inhibited by wortmannin [20, 21, 31, 42]. Several lines of evidence indicate that PI 3-kinase may be involved in various signal transduction events: activation of Ras [14], Rac [15], activation of PKCζ [16, 17], and actin polymerization [11, 18]. Rac appears to be involved in controlling Rho and actin polymerization, leading to the formation of lamellipodia and membrane ruffles [44]. It was demonstrated recently that Ras [45] and the Rho family (Rho, Rac, Cdc42) [25, 46–48] take part in modulating PLD activity. Taken together, we would speculate that PI 3-kinase activation leads to the activation of Ras and/or Rac (and subsequently Rho), which sequentially regulates the activity of PLD. However, to prove this hypothetical notion, further work is necessary and is currently in progress.

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