G-protein-stimulated Phospholipase D Activity Is Inhibited by Lethal Toxin from Clostridium sordellii in HL-60 Cells

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Lethal toxin (LT) from Clostridium sordellii has been shown in HeLa cells to glucosylate and inactivate Ras and Rac and, hence, to disorganize the actin cytoskeleton. In the present work, we demonstrate that LT treatment provokes the same effects in HL-60 cells. We show that guanosine 5′-O-(3-thiotriphosphate)-stimulated phospholipase D (PLD) activity is inhibited in a time- and dose-dependent manner after an overnight treatment with LT. A similar dose response to the toxin was found when PLD activity was stimulated by phorbol 12-myristate 13-acetate via the protein kinase C pathway. The toxin effect on actin organization seemed unlikely to account directly for PLD inhibition as cytochalasin D and iota toxin from Clostridium perfringens E disorganize the actin cytoskeleton without modifying PLD activity. However, the enzyme inhibition and actin cytoskeleton disorganization could both be related to a major decrease observed in phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). Likely in a relationship with this decrease, recombinant ADP-ribosylation factor, RhoA, Rac, and RalA were not able to reconstitute PLD activity in LT-treated cells permeabilized and depleted of cytosol. Studies of phosphoinositide kinase activities did not allow us to attribute the decrease in PtdIns(4,5)P2 to inactivation of PtdIns4P 5-kinase. LT was also found to provoke a major inhibition in phosphatidylinositol 3-kinase that could not account for the inhibition of PLD activity because wortmannin, at doses that fully inhibit phosphatidylinositol 3-kinase, had no effect on the phospholipase activity. Among the three small G-proteins, Ras, Rac, and RalA, inactivated by LT and involved in PLD regulation, inactivation of Ral proteins appeared to be responsible for PLD inhibition as LT (strain 9048) unable to glucosylate Ral proteins did not modify PLD activity. In HL-60 cells, LT treatment appeared also to modify cytosol components in relationship with PLD inhibition as a cytosol prepared from LT-treated cells was less efficient than one from control HL-60 cells in stimulating PLD activity. Phosphatidylinositol transfer proteins involved in the regulation of polyphosphoinositides and ADP-ribosylation factor, a major cytosolic PLD activator in HL-60 cells, were unchanged, whereas the level of cytosolic protein kinase Ca was decreased after LT treatment. We conclude that in HL-60 cells, lethal toxin from C. sordellii, in inactivating small G-proteins involved in PLD regulation, provokes major modifications at the membrane and the cytosol levels that participate in the inhibition of PLD activity. Although Ral appeared to play an essential role in PLD activity, we discuss the role of other small G-proteins inactivated by LT in the different modifications observed in HL-60 cells.

Phospholipase D (PLD) hydrolyzes phosphatidylcholine, the major membrane phospholipid, yielding choline and phosphatic acid (PA). PA, a possible second messenger, is fusogenic, and its increase is associated with several important cellular modifications such as activation of protein kinases, phospholipase C-γ and cytosolic phospholipase A2 (cPLA2), Ca2+ influx, DNA synthesis, and c-fos and c-myc transcription. In cells, PA is rapidly converted by a PA phosphohydrolase into diacylglycerol, the natural activator of protein kinase C (PKC), and hence, PLD activity can lead to a long term activation of protein kinase C (see review Ref. 1).

In mammalian cells, PLD is stimulated via several membrane receptors. Its activation can occur by different intracellular pathways. Over the past few years, several components participating in the control of PLD activity have been deciphered. Small G-proteins, including ARF which is involved in membrane traffic and RhoA which participates in actin polymerization, were demonstrated to be potent activators of PLD. Other small G-proteins implicated in the regulation of actin polymerization, namely Rac and Cdc42, were also shown to be PLD activators but to a lesser extent (see review Ref. 2). PLD activation was also reported to occur through a Ras-Ral pathway (3–6); activated RalA leads to membrane recruitment of ARF (3, 4). Other PLD activators have been identified, such as conventional PKC isoforms (see review Ref. 2) and the actin-binding protein gelsolin (7). PLD is under the negative control of several proteins, one of which was demonstrated to be the cytoskeletal protein, fodrin, an actin-binding protein (8) and two others were identified as synaptotagmin (9) and clathrin.

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assembly protein 3 (10). Moreover, phosphoinositides are also involved in PLD regulation. In particular, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), the PtdIns-phospholipase C substrate, was shown to be a necessary cofactor for PLD activity (11). How all the components involved in the regulation of PLD activity interact in resting or activated cells is not yet well understood.

Bacterial protein toxins of large molecular weight isolated from Clostridium difficile (toxins A and B) have been shown to inactivate Rho proteins and, thus, to affect the actin cytoskeleton (12). Toxin B has Rho subfamily small G-proteins, including RhoA, Rac, and Cdc42, as targets. It inactivates these proteins by modifying Rho by a glucosylation on threonine 37 and Rac and Cdc42 on threonine 35 (12). This toxin was demonstrated to inhibit m3 muscarinic receptor stimulation of PLD in human embryonic kidney (HEK) cells (13) by decreasing the membrane level in PtdInsP2 (14).

Clostridium sordelli, the organism responsible for gas gangrene, produces a large molecular weight toxin, lethal toxin (LT), which exhibits some similarities with toxin B (15). LT also inactivates small G-proteins by having a glucosyltransferase activity leading to the glucosylation of small G-proteins (Ras, Rac, Rap, and Rap). In vitro glucosylation was performed on a cell lysate containing 320 µg of proteins per assay in a final volume of 300 µl. At the end of the reaction (1 h at 37 °C), bovine serum albumin (1 mg/ml final) and (IP82) and (9048) strains, and UDP-[3H]glucosylase with 5 mg/ml LT in glucosylation buffer, pH 7.5, made of 50 mM triethanolamine, 2 mM MgCl2, 1 mM EGTA, and 0.1 mM GDP, and protease inhibitor mixture (Complete™) according to instructions. Cells were incubated on ice for 20 min, and complete lysis was achieved by three cycles of freeze-thawing. Cell lysates were centrifuged at 400 × g to remove intact cells and nuclei, and the amount of protein in each superantigen was determined. For in vitro glucosylation of small GTP-binding proteins, 32 µg of cell lysate or 2 µg of purified small G-proteins was added to 3 µl of dried UDP[3H]glucosylase with 5 mg/ml LT in glucosylation buffer, pH 7.5, made of 50 mM triethanolamine, 2 mM MgCl2, 1 mM EDTA, and 0.03 mM GDP. The reaction was carried out for 1 h at 37 °C and stopped by adding 10 µl of sample buffer (3 times). Immunoprecipitates were analyzed by SDS-PAGE and autoradiographed as described above.

Immunoprecipitation of Small G-proteins

In vitro glucosylation was performed on a cell lysate containing 320 µg of proteins per assay in a final volume of 300 µl. At the end of the reaction (1 h at 37 °C), bovine serum albumin (1 mg/ml final) and (IP82) and (9048) strains, and UDP-[3H]glucosylase with 5 mg/ml LT in glucosylation buffer, pH 7.5, made of 50 mM triethanolamine, 2 mM MgCl2, 1 mM EGTA, and 0.1 mM GDP, the reaction was carried out for 1 h at 37 °C and stopped by adding 10 µl of sample buffer (3 times). Immunoprecipitates were analyzed by SDS-PAGE and autoradiographed as described above.

**Immunoprecipitation of Small G-proteins**

**Materials**

RPML 1640 and medium 199 were purchased from Life Technologies, Inc. Fetal bovine serum was from Daccher. Radiolabeled molecules, [methyl-3H]-choline chloride (82 Ci/mmol), [myo-3H]-inositol (16.5 Ci/mmol), were obtained from Amersham Pharmacia Biotech. 1-O-alkyl1,2,3-H]lysoPAF(30–60 Ci/mmol), 1-dipalmitoyl, [choline,methyl-3H]-30–60 Ci/mmol), [γ-32P]ATP (5000 Ci/mmol), and ENHANCE spray were purchased from NEN Life Science Products. Streptolysin O was from Murex Diagnostic Ltd. GTPγS, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), protease inhibitor mixture table (Complete™), and protease and fatty acid-free bovine serum albumin were purchased from Roche Molecular Biochemicals (Germany). Bio-Rex 70 cation-exchange resin was purchased from Bio-Rad. LY 294002 was obtained from Calbiochem. Cytochalasin D, wortmannin, FITC phalloidin, protein A-agarose beads and all other chemical products were from Sigma. Scintillant liquid, Optiphase “HiSafe” 3, was from EG & G. Silica gel 60 plates for TLC analysis were obtained from Whatman. Anti-Ha-Ras (259) and anti-Rac (T-17) were from Santa Cruz Biotechnology. Rabbit anti-IgG and anti-goat IgG were from Cappel. ARF antibodies were obtained after immunization of rabbits with recombinant ARF.

Lethal toxin and iota toxin were purified to homogeneity from C. sordelli (IPS2) and (9048) strains and Clostridium perfringens E, respectively, as extensively reported elsewhere (19, 20). Toxin B from C. difficile was prepared according to von Eichel-Streiber et al. (21). Recombinant ARF, RhOA, Rac1, and RapA were made in Escherichia coli through glutathione S-transferase fusions and were prepared by thrombin digestion to yield the 21-KDa proteins.

**Methods**

Cells—HL-60 cells were cultured in suspension in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 2% l-glutamine at 37 °C in a humidified incubator containing 5% CO2.

Glucosylation by Lethal Toxin of Small GTP-binding Proteins in HL-60 Cells—HL-60 cells grown in RPMI 1640 medium were treated by LT from C. sordelli as indicated. After treatment, cells (2 × 107) were washed 6 times in washing buffer before addition of 8 µl of sample buffer (3 times). Fluorescence experiments were carried out in intact cells or in permeabilized cells. For experiments made with permeabilized cells or PtdEt formed in the presence of a short chain alcohol, instead of PA, a stable phosphatidyl alcohol will be formed. Measurement of phosphatidyl alcohol (Ptd-alcohol) in intact cells was performed according to instructions. Cells were incubated on ice for 20 min, and complete lysis was achieved by three cycles of freeze-thawing. Cell lysates were centrifuged at 400 × g to remove intact cells and nuclei, and the amount of protein in each superantigen was determined. For in vitro glucosylation of small GTP-binding proteins, 32 µg of cell lysate or 2 µg of purified small G-proteins was added to 3 µl of dried UDP-[3H]glucosylase with 5 mg/ml LT in glucosylation buffer, pH 7.5, made of 50 mM triethanolamine, 2 mM MgCl2, 1 mM EDTA, and 0.03 mM GDP. The reaction was carried out for 1 h at 37 °C and stopped by adding 10 µl of sample buffer (3 times). Immunoprecipitates were analyzed by SDS-PAGE and autoradiographed as described above.

Fluorescence Experiments, Effect of LT on the Actin Cytoskeleton

After treatment with LT, cells were fixed with 3% paraformaldehyde for 15 min and then washed three times with PBS. Free aldehyde groups were quenched in NH4Cl (50 mM) in PBS containing CaCl2 and MgCl2 (1 µM) for 10 min. Cells were permeabilized for 5 min with Triton X-100 0.3% in PBS for 5 min and then incubated with FITC-phalloidin for 30 min. After labeling, cells were washed three times in PBS, fixed on slides covered with polylysine, and mounted in Mowiol. All steps were carried out at room temperature. Fluorescent images were taken with a confocal laser scanning microscope (Bio-Rad MRC-1000) attached to a diapath 300 microscope (Nikon). For fluorescence detection, a krypton/argon laser at 488 excitation was used, and emission was collected with a 522-nm filter.

**Determination of Phospholipase D Activity in Permeabilized Cells and in Intact Cells**

In HL-60 cells, PLD hydrolyzes preferentially phosphatidylcholine, the major phospholipid, to give choline and phosphoric acid, both can reflect PLD activity but can also result from other cellular activities. PLD possesses a specific transphosphatidylation activity (22); in the presence of a short chain alcohol, instead of PA, a stable phosphatidyl alcohol will be formed. Measurement of phosphatidyl alcohol (Ptd-alcohol) is highly specific of PLD in comparison to choline measurement. We have previously checked that the formation of both products of PC hydrolysis, Ptd-alcohol and choline, both occurred in parallel and reflected PLD activity in streptolysin O-permeabilized HL-60 cells (23). Thus PLD activity was estimated by measuring choline formed in experiments made with permeabilized cells or PtdEt formed in the presence of 1.5% ethanol in intact cells.

**Choline Measurement—**Cells were labeled at isotopic equilibrium for 48 h in medium 199 containing 10% fetal bovine serum, the presence of 0.5 µCi/ml [3H]choline chloride. At the end of labeling, HL-60 cells were counted, and viability was monitored by trypan blue exclusion. Cells were washed and resuspended at a concentration of 107 cells/ml in a buffer made of 137 mM NaCl, 2.7 mM KCl, 20 mM PIPES, and 0.1% protease and fatty acid-free bovine serum albumin, pH 6.8 (PIPES buffer). Phospholipase D activity measurement was carried out in a final volume of 100 µl at 37 °C in the presence of MgCl2 (2 mM final
concentration), MgATP (2 mM final concentration), GTP-
S (25 \( \mu \)M final concentration), Ca\(^{2+}\) (10 \( -5 \) m), buffered with EGTA (3 mM final concentration), and streptolysin O (0.4 units/ml final concentration) as permeabilizing agent. The enzymatic reaction was started by addition of 50 \( \mu \)l of labeled cells (5 \( \times \)10\(^6\) cells) and stopped after 20 min at 37 \(^\circ\)C by addition of 500 \( \mu \)l of chloroform/methanol (1:1, \( v/v \)), and then 150 \( \mu \)l of water were added to extract the aqueous phase containing radiolabeled metabolites. The aqueous phase was loaded on columns of 1 ml of Bio-Rex 70 cation-exchange resin to separate newly formed choline from phosphorus-containing choline metabolites as described by Martin (24). Radioactivity present in choline was quantified after addition of scintillant liquid using a Wallac 1410 liquid scintillation counter. Total radioactivity associated with cells was counted. PLD activity was estimated by measuring choline formed and expressed as the percentage of total radioactivity incorporated in cells or as indicated.

Cytosol-depleted cells were obtained after permeabilization for 10 min at 37 \(^\circ\)C in the presence of 0.4 units/ml streptolysin O as described in detail elsewhere (23).

**PtdEt Measurement**—HL-60 cells were washed in a buffer made of 137 mM NaCl, 2.7 mM KCl, 20 mM Hapes, 5.6 mM glucose, and 0.1% protease and fatty acid-free bovine serum albumin, pH 6.8, and incubated at 37 \(^\circ\)C for 30 min in the same buffer containing 2 mM MgATP, 0.6 mg/ml PS (0.6 mg/ml) and 0.2 mM unlabeled MgATP and performed at room temperature. Incorporation of \(^{32}\)P-ATP into PtdIns4P and PtdIns(4,5)P\(_2\) was measured by 30–50% after that time, using \(^{32}\)P-labeled PtdIns4P and PtdIns(4,5)P\(_2\) as a tracer. After loading with PtdIns4P, cells, depleted of cytosol, were used for measurement of PLD activity in the presence of different small G-proteins as described above.

**Determination of PtdIns 4-Kinase and PtdIns(4)P 5-Kinase Activities**

Phosphatidylinositol 4-kinase (PtdIns 4-kinase) and phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4)P 5-kinase) activities were performed in a final volume of 200 \( \mu \)l containing 40 \( \mu \)l of HL-60 cell lysates in the presence of 10 \( \mu \)l of a sonicated solution containing PtdIns4P (1.4 mg/ml) and PS (0.6 mg/ml) as described by Chong et al. (26). The reaction was started by addition of 10 \( \mu \)l of buffer containing \(^{32}\)P-ATP (0.1 mM/cell) and 0.2 mM unlabeled MgATP and performed at room temperature. Incorporation of \(^{32}\)P into PtdIns(4)P and PtdIns(4,5)P\(_2\) was found to be linear for 6 min under such conditions. Five min after addition of the radioactive substrate, the reaction was stopped with 0.6 mM methanol, 1 \( \mu \)l HCl (1:1). Phospholipids were extracted by adding 0.5 ml of chloroform. The organic phase was dried under vacuum, and lipids were resuspended in chloroform (40 \( \mu \)l) were separated onto TLC plates according to Chong et al. (26). \(^{32}\)P-Labeled phosphoinositides were visualized by autoradiography and quantified using a molecular imager (Molecular Dynamics).

**Determination of PtdIns 3-Kinase Activity**

PtdIns 3-kinase activity was performed in 500 \( \mu \)l final volume under conditions similar to those described above for other PtdIns kinases except that the amount of radiolabeled ATP was equivalent to 0.25 mCi per sample. This enzyme activity was estimated by measuring the amount of D3-phosphorylated inositol lipids present in cells using an HPLC technique as described (27, 28). Analysis of the deacylated lipids
was performed as described (28). Radioactivity eluted from the Partisphere SAX column (Whatman International, Maidstone, Kent, UK) was monitored and quantified by LB 506C detector (Berthold, Munich, Germany), using the Cerenkov effect.

**HL-60 Cell Cytosol Preparation**

HL-60 cells (10^6) were washed 3 times in isotonic PIPES buffer, pH 6.8, and resuspended in 3 ml of hypotonic buffer made of Tris (20 mM), MgCl₂ (5 mM), EDTA (1 mM), sucrose (250 mM), orthovanadate (0.1 mM), DTT (1 mM), and protease inhibitor mixture (Complete™TM), pH 7.5. After 30 min incubation on ice, cells were homogenized with a Dounce homogenizer (50 strokes) followed by three cycles of freeze-thawing. Unbroken cells and nuclei were removed by a 5-min centrifugation at 400 × g. Particulate fractions were pelleted and removed from cytosol by centrifugation for 20 min at 300,000 × g in a TI100 ultracentrifuge (Beckman). Cytosol extracts were tested for their ability to reconstitute PLD activity in cytosol-depleted permeabilized cells.

**Immunoblot Analysis**

After SDS-PAGE, proteins were transferred electrophoretically onto a nitrocellulose membrane. Blocking was achieved overnight at 4 °C in isotonic PBS/glycine (2%) with 3% non-fat dry milk. Western blot analysis was performed for 90 min at room temperature using a polyclonal anti-recombinant ARF antibody (1:500 dilution) in the same buffer containing 10% FBS and 0.3% Tween 20. The blots were then incubated for the same period with 125I-protein A (0.2 μCi/ml). Immunoblots were analyzed with a PhosphorImager (Molecular Dynamics) for quantitative evaluation of the radioactivity in bands.

Western blot analysis with anti-PITP antibodies and protein quantification were kindly performed as described (29).
dead cells was increased to 20–30%. Thus, we tested the effect of 100 ng/ml LT on the cytoskeleton in this cell line. As shown in Fig. 2, overnight treatment of HL-60 cells with LT induced a neat decrease in cortical actin polymerization, indicating the efficiency of the treatment.

LT Inhibits PLD Activity—As LT glucosylates several small G-proteins reported to be involved in PLD regulation, we then studied the effect of LT on PLD activity. As reported in Fig. 3, LT inhibits PLD activity in a time- and dose-dependent manner. In HL-60 cells, GTPγS-stimulated PLD activity, measured in streptolysin O-permeabilized HL-60 cells, was decreased by 20–30% after 6 h of treatment with LT (100 ng/ml), and maximal inhibition (50–60% of the control) was reached after 16 h (Fig. 3A). This PLD activity was inhibited in a dose-dependent manner after an overnight treatment of HL-60 cells with LT. Maximal effect that reduces GTPγS-stimulated PLD activity by 60% was obtained with 30 ng/ml. A slight decrease in basal PLD activity was also observed at the highest LT dose studied (100 ng/ml) (Fig. 3B), and PMA-stimulated PLD activity, measured in intact cells, was inhibited to the same extent by similar doses of LT (Fig. 3C).

Effect of LT Treatment on Reconstitution of GTPγS-stimulated PLD Activity with Small G-proteins in Cytosol-depleted HL-60 Cells—We have already shown that HL-60 cells permeabilized with streptolysin O can be depleted of cytosol (23). Thus, cytosol-depleted cells represent an accurate model to study whether the decrease in PLD activity, observed after LT treatment, is related to a change occurring at the membrane or

TABLE I

| Cell Treatment       | PLD Stimulation       | PLD Activity (%) of PtdCho Hydrolysis
|----------------------|-----------------------|------------------------------------------
|                      | No GTPγS             | GTPγS (25 µM)                         |
| Control              | 0.51 ± 0.08          | 1.13 ± 0.80                           |
| Lethal toxin (100 ng/ml) | 0.30 ± 0.10          | 0.50 ± 0.03                           |
| Toxin B (15 ng/ml)   | 0.19 ± 0.04          | 0.21 ± 0.01                           |
| ia-ib (100 ng/ml)    | 0.34 ± 0.11          | 0.79 ± 0.08                           |
| Cytochalasin D (1 µM)| 0.50 ± 0.10          | 1.10 ± 0.40                           |

Effect of different molecules modifying the actin cytoskeleton on PLD activity in HL-60 cells depleted of cytosol

HL-60 cells were labeled with [3H]choline chloride (0.5 µCi/ml) for 48 h in medium 199 and treated overnight with different molecules before PLD activity measurement. The enzymatic reaction was carried out in the presence or not of GTPγS and small G-proteins (ARF, RhoA). The values in the table represent PLD activity measured in triplicate and expressed as the percentage of PtdCho hydrolysis ± S.E. This experiment is representative of three different ones.
the cytosol level. The first possibility was tested using LT-treated and control HL-60 cells depleted of cytosol after permeabilization. PLD activity was then stimulated in the presence of GTPγS by different small G-proteins known to be PLD activators as follows: ARF and RhoA which cannot be modified regardless which concentration of LT was used (data not shown). These results do not exclude an indirect link between LT effect on the actin cytoskeleton and on GTPγS-stimulated PLD activity such as a change in phosphoinositides required for actin polymerization and for PLD activation.

LT Treatment Decreases Polyphosphoinositides Levels; Its Relationship between LT Effect on the Cytoskeleton and PLD Inhibition—To study if the decrease in PLD activity provoked by LT treatment was directly related to actin polymerization, we studied the effect of different molecules modifying the cytoskeleton on PLD activity. As reported in Table I, overnight treatment of cells with toxin B (15 ng/ml, overnight) from C. difficile was able to inactivate PLD in HL-60 cells to an even greater extent than LT; this toxin was reported to modify the actin cytoskeleton in different cell types. However, cytochalasin D (1 μM/ml, overnight) which disrupts the cytoskeleton and iota toxin (100 ng/ml, overnight) which depolymerizes actin (31) did not provoke marked changes in PLD activity in cytosol-depleted cells stimulated with ARF or RhoA. In HL-60 cells, the effect on the actin cytoskeleton of toxin B, cytochalasin D, and iota toxin was checked; they all provoked a clear decrease in cortical polymerized actin (data not shown). These results do not exclude an indirect link between LT effect on the actin cytoskeleton and on GTPγS-stimulated PLD activity such as a change in phosphoinositides required for actin polymerization and for PLD activation.

Since PtdIns(4,5)P2 (PtdInsP2) is a cofactor for ARF-stimulated PLD activity, we studied the effect of LT treatment on phosphoinositide composition. As reported in Fig. 5, overnight treatment of HL-60 cells induced modifications in polyphosphoinositide levels in the range of LT concentrations that provoked inactivation of ARF- and RhoA-stimulated PLD activity. PtdInsP (Fig. 5A) and PtdInsP2 (Fig. 5B) levels were both decreased by LT in a dose-dependent manner. At 30 ng/ml, a dose shown to inhibit maximally small G-protein-stimulated PLD activity, PtdInsP and PtdInsP2 were decreased by about 30 and 50%, respectively. PtdIns level was not modified regardless which concentration of LT was used (data not shown).

Since PtdIns(4,5)P2 (PtdInsP2) is a cofactor for PLD activity, a decrease in the level of this minor polyphosphoinositide could be responsible for the inability of small G-proteins such as ARF and RhoA to stimulate PLD activity fully in LT-treated cells. Therefore, the effect of PtdInsP2 addition was tested on small G-protein-stimulated PLD activity. PLD activity stimulated with ARF, RhoA, Rac, or RalA in the presence of GTPγS was measured in untreated and LT-treated cells. As shown in Fig. 6, control and LT-treated cells loaded with PtdInsP2 responded slightly better to each small G-protein. However, PtdInsP2 was not able to restore fully GTPγS- and small G-protein-stimulated PLD activity in LT-treated HL-60 cells.

As both Rac and Ras have been reported to participate in in PtdIns4P 5-kinase and PtdIns3-kinase regulation responsible for PLD cofactor synthesis, the effect of LT treatment was examined on polyphosphoinositide kinases.

PtdIns 4-kinase and PtdIns4P 5-kinase activities were measured under conditions similar to PLD activity measurement, in
Lethal Toxin Effects on Phospholipase D in HL-60 Cells

Involvement of Ral in the Inhibition of GTPγS-stimulated PLD Activity—RalA, also inactivated by LT-IP82, has been reported to be essential for maintaining the level of PtdInsP2 (32) and ARF and PKCα, potent activators of PLD. Thus, the level of these proteins was measured in the cytosol recovered from untreated and LT-treated cells. As reported in Fig. 11, A and B, no modification in the level of PITP and ARF was observed after LT treatment. In contrast, cytosolic PKCα decreased by about 40% in LT-treated cells (Fig. 11C). Quantification of the bands by scan densitometer or PhosphorImager showed that the amount of PITP and ARF differed by around 6% (decrease for PITP and increase for ARF) between control and LT-treated cells, and PKC was decreased by 61% in LT-treated cells by comparison to untreated cells.

In the present work, we have investigated different effects of lethal toxin (LT) purified from C. sordelli (strain IP-82) in HL-60 cells. Toxins purified from different Clostridia origins have been demonstrated to inactivate small G-proteins of the Rho family, and target profiles of their intracellular activity are specific for each of them. It has been reported that, in HeLa

the absence and in the presence of GTPγS. As shown in Fig. 7, after overnight treatment of cells with 100 ng/ml LT, PtdIns 4-kinase was markedly increased by about 200% compared with untreated cells (Fig. 7A), whereas PtdIns4P 5-kinase (Fig. 7B) was not modified either with or without GTPγS. Therefore, the decrease in PtdInsP and PtdInsP2 levels cannot be related to a change in the activity of the kinases involved in their synthesis.

PtdIns 3-kinase activity was measured in control and LT-treated cells. As reported in Table II, the activity of this enzyme was profoundly inhibited after cell treatment with LT, as estimated by the amount of the different polyphosphoinositide isoforms phosphorylated in position 3 of inositol lipids identified by HPLC analysis. In LT-treated cells, phosphoinositides generated by PtdIns 3-kinase represent 15–20% of those in control cells. Wortmannin, a specific PtdIns 3-kinase inhibitor, decreased the enzyme activity by 95% in HL-60 cells when used at 20 μM for 10 min (conditions known to specifically inhibit PtdIns 3-kinase activity).

Relationship between LT Inactivation of Ras and PLD Inhibition—To check if the observed decrease in PtdIns 3-kinase activity could account for PLD inhibition, HL-60 cells were treated for 20 min with 20 and 50 nM wortmannin. No inhibition of PLD activity was observed (Fig. 8) whichever the concentration used. Similar results were obtained using LY 294002, another specific PtdIns 3-kinase inhibitor (data not shown). Therefore, it is unlikely that inhibition of PLD by LT would be related to Ras/PtdIns3-kinase inhibition in this model.

Involvement of Ral in the Inhibition of GTPγS-stimulated PLD Activity—RalA, also inactivated by LT-IP82, has been reported to take part in PLD activation. To study the role of this small G-protein on GTPγS-stimulated PLD activity, we used a different lethal toxin extracted from C. sordelli, strain 9048, that does not possess the capacity to glucosylate Ral in vitro but is still able to inactivate Ras and Rac (Fig. 9A). As reported in Fig. 9B, GTPγS-stimulated PLD activity in HL-60 cells was not modified after a treatment with 1 μg/ml LT-9048, whereas it was decreased by about 50% when cells were treated with only 0.1 μg/ml of LT-IP82.

Inhibition of GTPγS-stimulated PLD Activity by LT Treatment also Involves a Modification at the Cytosol Level in HL-60 Cells—The possibility that LT treatment could have an effect on a factor present in the cytosol was also investigated. For this purpose, cytosolic fractions from control and LT-treated cells were prepared, and their respective ability to stimulate PLD activity in the absence of GTPγS was tested in control HL-60 cells that were depleted of cytosol by a pre-permeabilization step. As reported in Fig. 10A, cytosol (60 μg of proteins/assay) extracted from LT-treated cells did not stimulate GTPγS-dependent PLD activity in control cells to the same extent as cytosol extracted from untreated cells. This result indicates that a cytosolic factor involved in PLD activity is either missing or decreased after LT treatment.

Several proteins involved in PLD regulation are known to be cytosolic including phosphatidylinositol transfer protein (PITP) reported to be essential for maintaining the level of PtdInsP2 (32) and ARF and PKCα, potent activators of PLD. Thus, the level of these proteins was measured in the cytosol recovered from untreated and LT-treated cells. As reported in Fig. 11, A and B, no modification in the level of PITP and ARF was observed after LT treatment. In contrast, cytosolic PKCα was decreased by about 40% in LT-treated cells (Fig. 11C). Quantification of the bands by scan densitometer or PhosphorImager showed that the amount of PITP and ARF differed by around 6% (decrease for PITP and increase for ARF) between control and LT-treated cells, and PKC was decreased by 61% in LT-treated cells by comparison to untreated cells.

DISCUSSION

In the present work, we have investigated different effects of lethal toxin (LT) purified from C. sordelli (strain IP-82) in HL-60 cells. Toxins purified from different Clostridia origins have been demonstrated to inactivate small G-proteins of the Rho family, and target profiles of their intracellular activity are specific for each of them. It has been reported that, in HeLa
cells, LT enters cells by endocytosis and, after toxin entry, cells round up as a consequence of F-actin reorganization since disruption of actin stress fibers and formation of filopodia occur. Possessing a glucosyltransferase activity, LT monoglucosylates on threonine 35 of Ras and Rac, Rac and Rho in vitro (16, 18). Under their GTP-bound form, all these small G-proteins except Rap have been reported to participate in PLD activation (5, 6, 33).

In the present study, we observed that LT treatment provokes, in HL-60 cells, glucosylation of small G-proteins, among which are Rac and Ras, modification of the actin cytoskeleton, as in HeLa cells (16), and a decrease in PLD activity stimulated by GTPyS in permeabilized cells and by PMA in intact cells. In this cell line, GTPyS is known to provoke a major activation of PLD increasing basal activity by a factor 8 to 10 in the presence of optimal concentrations of Mg-ATP and Ca²⁺ (34). In all our attempts to measure PLD activity using artificial vesicles with various phospholipid compositions as the enzyme substrate, addition of sodium oleate to vesicles provoked a major decrease in phosphatidylcholine breakdown in this cell line.² These results indicate that HL-60 cells contain little or no oleate-activated PLD. These cells have been shown to express high levels of mRNA for PLD1, particularly, and PLD2 (35), both isoforms requiring PtdIns(4,5)P₂ as a cofactor for activity (36, 37). The former human isoform can be directly activated in vitro by ARF, Rho protein family, and PKCα (33), and the latter was recently reported to be stimulated by ARF also (38). A direct interaction between the carboxyl terminus of PLD and activated RhoA using the two-hybrid technique has been reported by Sung et al. (39). So far, no direct interaction between PLD and ARF was reported; this could be due to a pre-required binding of ARF and/or PLD to PtdIns(4,5)P₂ (40, 41) for interaction.

In HL-60 cells, PLD activity was shown to be calcium-dependent (34). In these promyelocytic cells, LT inhibits PLD activity, whichever its pathway of stimulation, and hence its effect is partly different to that observed in an epithelial cell line, HEK. Indeed, in a recent work, Schmidt et al. (42) found that LT was able to decrease exclusively the PKC pathway of PLD activation in HEK-293 cells stably expressing m3 muscarinic receptors by inactivating Rac proteins. In HL-60 cells, Rac proteins also appeared to be the major target for LT effect on PLD activity. Indeed, LT extracted from strain 9048 of C. sordellii, which does not inactivate Rac proteins in vitro, was not able to provoke modification in PLD activity in these cells.

In HL-60 cells, LT (from strain IP-82) was found to generate major changes in cytoskeleton organization, membrane phospholipid composition, and cytosolic PKCα level. These profound modifications could be related to the concomitant alteration in PLD activity stimulated via different signaling pathways.

LT treatment induced an almost complete disorganization of cortical actin cytoskeleton in HL-60 cells. In HeLa cells, cytoskeletal modifications induced by this toxin were reported to be different from those provoked by toxin B from C. difficile. Such differences can be due to their different major targets in vivo, Ras and Rac for LT and Rho proteins and RhoA, Rac, and Cdc42 for toxin B. All these small G-proteins have been reported to exert a control on the actin cytoskeleton through different pathways (43, 44). In HL-60 cells, which grow in suspension, a similar modification was observed with both toxins, a major decrease in polymerized actin. LT could modify the actin cytoskeleton in inactivating Rac and/or Rac. Several studies suggest an implication of the cytoskeleton in PLD regulation. Small G-proteins of the Rho subfamily, well established to be involved in the regulation of actin polymerization, are also activators of PLD (33, 45), and RhoA was reported to interact directly with PLD1 (39). The actin-binding protein, fodrin, was found to inhibit PLD activity (8), and gelsolin was shown to interact with PLD and activate the enzyme (7).

From the present study, the major decrease in actin polymerization observed after LT treatment in HL-60 cells is likely to be related to Ras/PtdIns 3-kinase/Rac pathway(s), but changes at the cytoskeleton level do not appear to be directly responsible for PLD inhibition. In HL-60 cells, we observed that LT treatment, giving a depolymerization in cortical actin, was accompanied by a profound decrease in PtdIns 3-kinase activity, a direct target for Ras (46). Indeed, control of actin cytoskel-

² V. Montalescot and B. Geny, unpublished results.
**TABLE II**

**PtdIns 3-kinase activity in HL-60 cells treated with lethal toxin from C. sordelli**

After labeling and stimulation of HL-60 cells as detailed under “Experimental Procedures,” phosphoinositides labeled in position 3 were measured by HPLC. Phosphatidylinositol 3-kinase was specifically inhibited by treating cells for 10 min with 20 nm wortmannin. The effect of LT (100 ng/ml) on D3-phosphoinositide formation was measured after an overnigth treatment. Results are given in dpm, and the experiment shown is representative of two.

| Cell treatment          | PtdIns3P | PtdIns3,4P | PtdIns3,4,5P |
|-------------------------|---------|-----------|--------------|
| None (control)          | 1615    | 4125      | 881          |
| Lethal toxin (LT), overnight, 100 ng/ml | 50      | 891       | 167          |
| Wortmannin, 10 min, 20 ng/ml | 0       | 158       | 129          |

**FIG. 8.** Effect of wortmannin, a PtdIns3-kinase inhibitor on PLD activity. [3H]Choline-labeled cells were washed and incubated at 37 °C for 20 min in the absence or in the presence of the indicated concentrations of wortmannin; [3H]choline release was then measured in the absence (open bars) and in the presence (dotted bars) of GTPγS (25 μM). PLD activity was expressed as the percentage of total [3H]choline incorporated in cells present in choline. In the experiment shown, the total radioactivity in cells was 164,639 ± 5,398 dpm. This experiment was repeated three times with similar results.

**FIG. 9.** Effect of lethal toxin from strain 9048 of *C. sordelli* on small G-protein glucosylation *in vitro* and on GTPγS-stimulated PLD activity. A, recombinant Ras, Rac, and Ral were glucosylated *in vitro*, as in Fig. 1, in the presence of LT extracted from strain 9048 of *C. sordelli*. B, [3H]choline-labeled HL-60 cells were treated or not treated overnight with LT from strains 9048 and IP-82. [3H]Choline release was measured in the absence (open bars) and in the presence (dotted bars) of GTPγS (25 μM) in permeabilized cells. Lane 1, not treated (control cells); lane 2, treated with 1000 ng/ml from strain 9048; and lane 3, treated with 100 ng/ml strain IP-82. PLD activity was expressed as the percentage of total [3H]choline incorporated in cells present in choline. In the experiment shown, the total radioactivity in cells was 145,500 ± 9,080 dpm. This experiment was repeated three times with similar results.

ealon by Ras has been related to PtdIns 3-kinase activity, and Rac was shown to function downstream of Ras/PtdIns 3-kinase (43). As it has been recently reported that gelsolin dissociation from actin filament is under the control of Rac (47), Rac inactivation by LT could also specifically depolymerize actin by blocking actin/gelsolin interaction, preventing actin uncapping and blocking nucleating activity at the barbed end of F-actin. Thus, inactivation of both Ras and Rac by LT are likely to be responsible for cortical actin disorganization in HL-60 cells.

However, a direct relationship between PLD activation and the actin cytoskeleton organization can be ruled out as neither PtdIns 3-kinase inhibition nor drugs such as cytochalasin D, which caps the barbed end of actin filaments, and iota toxin, which ADP-ribosylates the actin monomers (31), have an effect on PLD activation in HL-60 cells. A similar observation has been made by Schmidt et al. (13) in HEK cells. In these cells, cytochalasin B and toxin C2 from *Clostridium botulinum* both disorganize the actin cytoskeleton but do not inhibit GTPγS-stimulated PLD activity as C3 toxin does, which inactivates Rho proteins.

Treatment with LT (strain IP-82) was found to lead to a net decrease in polyphosphoinositides in HL-60 cells. Such changes in the levels of negatively charged phospholipids could explain both the decrease in polymerized actin and the inability for any PLD-activating small G-protein to stimulate fully PLD activity in LT-treated cells. Proteins participating in the cytoskeleton or in the regulation of actin polymerization including profilin, gelsolin, and fodrin bind PtdIns(4,5)P2 and PtdIns(3,4,5)P3 with high affinity (48). Therefore, a decrease in these minor polyphosphoinositides would lead to changes in actin organization. As PLD activity in HL-60 cells appears to be mostly due to PLD isoforms requiring PtdInsP2 as cofactors, the important decrease in polyphosphoinositides observed after LT treatment can explain the lack of PLD activation by small G-proteins such as ARF and RhoA that are not modified by LT. The function of ARF is related to its interaction with PtdIns(4,5)P2 (40) and has also been shown to be the small G-protein responsible for PLD activation involving RaIA (3). This latter small G-protein was shown not to activate directly PLD but to recruit ARF to membranes which, thus, would stimulate PLD activity (4). Thus, the inability to restore PLD activity with PtdIns(4,5)P2 in treated HL-60 cells could explain why RaIA and ARF to-
LT treatment was also found to decrease the amount of PKCα in HL-60 cells. Cytosols prepared from untreated (control) and LT-treated cells were electrophoresed, and after transfer onto nitrocellulose membrane, their respective content in PITP, ARF, and PKCα was analyzed using specific antibodies as reported under “Experimental Procedures.” This experiment was repeated twice with similar results.

In HL-60 cells, GTPγS- and PMA-stimulated PLD activities were decreased to a similar extent and with similar doses of LT. This toxin was shown to inhibit only the PMA pathway in HEK cells (42). The discrepancies observed in the effects of LT are likely to be due to the difference in cell types. In HL-60 cells, the effect of LT was similar to that obtained with another bacterial toxin, toxin B from *C. difficile*, in this cell line; GTPγS- and PMA-stimulated PLD activities were both inhibited, and addition of PtdIns(4,5)P2 was not able to modify PLD activity significantly (49). In contrast, in HEK cells, this latter toxin only inhibits receptor and G-protein-stimulated PLD activity (13), and PtdIns(4,5)P2 was able to restore membrane-localized PLD activity (14). HL-60 cells and HEK cells are likely to possess different contents in PLD isoforms and/or different PLD regulators participating in each pathway of activation. HL-60 cells were found to have a more marked GTPγS-stimulated PLD activity than HEK cells and do not possess a PLD activity measurable in the presence of sodium oleate or sodium cholate as HEK cells do (13, 14, 50). Moreover, inactivation of Ras proteins appears to be sufficient for LT to inhibit PLD activity; it is likely that other small G-proteins inactivated by the toxin exert additional effects which might have stronger consequences in HL-60 cells than in HEK cells. Indeed, in these latter cell lines, LT was found to inhibit specifically PMA-stimulated PLD activity in the absence of modification in c-PKC cellular levels but not a pathway involving G-proteins. In these cells, the same effect was observed with toxin B-1470 from *C. difficile* that does not glucosylate Ras. In HL-60 cells, LT treatment was also found to decrease the amount of PKCα in cell cytosol. Such a cytosol from LT-treated cells was found to be less efficient to stimulate PLD activity in control cells. The exact mechanism of action for PKC and Ca2⁺ in PLD activation is not clear. It has been reported that the regulatory domain of PKCα in the presence of Ca2⁺ or PMA is sufficient to stimulate PLD activity (50). However, it has also been reported that PKC activity could also play a role by phosphorylating a phospholipase D-related component in the plasma membrane (51). In HL-60 cells, previous studies have shown that both Ca2⁺ and PMA can stimulate PLD in synergy with GTPγS (34). Thus, the regulatory domain of a c-PKC in the presence of Ca2⁺ could be necessary as a starter for other activators such as ARF or Rh proteins to stimulate PLD activity and the net decrease in PKCα cytosolic content could be responsible, at least in part, for a diminution in the synergistic effect between small G-protein, PKC and/or Ca2⁺. The decrease in cytosolic PKCα level could be a consequence of Ras inactivation as this small G-protein and PKC interaction have been reported to be required in activation signaling pathways (52–56). In glucosylating and inactivating Ras and other small G-proteins downstream of it, LT could inactivate PKC and in HL-60 cells rapidly lead to its “down-regulation.”

In conclusion, we demonstrate that LT from strain IP-82 of *C. sordellii*, which inactivates several small G-proteins, inhibits GTPγS- and PMA-stimulated PLD activity similarly. Using LT extracted from strain 9048 of *C. sordellii* which has no effect on Ras proteins, the responsibility for PLD inhibition is essentially due to Ras although recombinant Ras could not reconstitute PLD activation after LT treatment. Major changes in the level of polyphosphoinositides, acting as PLD cofactors, and in the cytosolic level of PKCα, an important PLD activator, are also observed and could be related to the high sensitivity of HL-60 cells to inactivation by LT not only of Ras proteins but also Rac and Ras.

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