Effect of Rho and ADP-ribosylation Factor GTPases on Phospholipase D Activity in Intact Human Adenocarcinoma A549 Cells*

Phospholipase D (PLD) has been implicated as a crucial signaling enzyme in secretory pathways. Two 20-kDa guanine nucleotide-binding proteins, Rho and ADP-ribosylation factor (ARF), are involved in the regulation of secretion and can activate PLD in vitro. We investigated in intact (human adenocarcinoma A549 cells) the role of RhoA and ARF in activation of PLD by phorbol 12-myristate 13-acetate, bradykinin, and/or sphingosine 1-phosphate. To express recombinant Clostridium botulinum C3 exoenzyme (using double subgenomic recombinant Sindbis virus C3), an ADP-ribosyltransferase that inactivates Rho, or dominant-negative Rho containing asparagine at position 19 (using double subgenomic recombinant Sindbis virus Rho19N), cells were infected with Sindbis virus, a novel vector that allows rapid, high level expression of heterologous proteins. Expression of C3 toxin or Rho19N increased basal and decreased phorbol 12-myristate 13-acetate-stimulated PLD activity. Bradykinin or sphingosine 1-phosphate increased PLD activity with additive effects that were abolished in cells expressing C3 exoenzyme or Rho19N. In cells expressing C3, modification of Rho appeared to be incomplete, suggesting the existence of pools that differed in their accessibility to the enzyme. Similar results were obtained with cells scrape-loaded in the presence of C3; however, results with virus infection were more reproducible. To assess the role of ARF, cells were incubated with brefeldin A (BFA), a fungal metabolite that disrupts Golgi structure and inhibits enzymes that catalyze ARF activation by accelerating guanine nucleotide exchange. BFA disrupted Golgi structure, but did not affect basal or agonist-stimulated PLD activity, i.e., it did not alter a rate-limiting step in PLD activation. It also had no effect on Rho-stimulated PLD activity, indicating that RhoA action did not involve a BFA-sensitive pathway. A novel PLD activation mechanism, not sensitive to BFA and involving RhoA, was identified in human airway epithelial cells by use of a viral infection technique that preserves cell responsiveness.

Phospholipase D (PLD), an important effector in receptor-mediated signal transduction pathways, catalyzes the hydrolysis of the most abundant membrane phospholipid, phosphatidylcholine, to generate choline and phosphatic acid. In intact cells, choline is rapidly phosphorylated to phosphocholine, which plays a role in cell proliferation (1). Phosphatic acid also serves as a second messenger in the regulation of secretion, DNA synthesis, and cell proliferation (2). PLD activity appears to be regulated, and can be activated by both phosphatidylinositol 4,5-bisphosphate (3) and phosphatidylinositol 1,4,5-trisphosphate (4) in vitro. Phosphatidylinositol 4,5-bisphosphate was essential for PLD activation in intact cells (5). Consistent with a regulatory role for protein kinase C (PKC), phorbol esters markedly activated PLD (6, 7), and PKC inhibitors abolished agonist-induced PLD activity (8) in some cells.

Both native and recombinant PLD were activated by ~20-kDa guanine nucleotide-binding proteins including ADP-ribosylation factors (ARFs) (3), which are critical for vesicular trafficking (9), and Rho, a GTPase that regulates several cellular functions including cytoskeletal organization (10). RhoA was required for guanosine 5′-O-(3-thio)triphosphate stimulation of PLD activity in neutrophil membranes (11). In rat fibroblasts, Clostridium botulinum C3 exoenzyme, which ADP-ribosylates and inactivates RhoA (12), inhibited activation of PLD by lysophosphatic acid (13). Similarly, toxin B from Clostridium difficile, which inactivates Rho family GTPases by monoglucosylation (14), abolished carbachol activation of PLD in HEK cells (15) and blocked PLD activation initiated via the IgE receptor in rat basophilic leukemia cells (16). More recently, it was demonstrated that activation of PLD via α2-adrenergic receptors in broken PC12 cell preparations required RhoA and PKC activation (17).

ARF restored guanosine 5′-O-(3-thio)triphosphate-dependent activation of PLD in cells depleted of cytosolic components (11, 18) and activated PLD associated with partially purified Golgi membranes (19). ARF proteins have also been implicated in the activation of PLD by insulin (20). Brefeldin A (BFA), a fungal metabolite that causes disruption of Golgi structure, can block ARF activation by inhibiting the guanine nucleotide-exchange protein that accelerates replacement of ARF-bound GDP by GTP (21–23). This causes dissociation of ARF and βCOP from Golgi membranes (in vivo and in vitro) before

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§The abbreviations used are: PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; BK, bradykinin; SPP, sphingosine 1-phosphate; PtdEtOH, phosphatidylethanol; ARF, ADP-ribosylation factor; dsSIN, double subgenomic recombinant Sindbis virus; CAT, chloramphenicol acetyltransferase; MOI, multiplicity of infection; PKC, protein kinase C; PBS, phosphate-buffered saline; IL, interleukin; BFA, brefeldin A.

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redistribution of Golgi components to the endoplasmic reticulum (24). BFA inhibited carbachol stimulation of PLD activity in HEK cells expressing muscarinic M3 receptors (25), but not PMA stimulation of PLD in HL-60 cells (26). Although BFA treatment has been used frequently to identify effects in cells that are ARF-dependent, the absence of BFA inhibition does not exclude a role for ARF, which can also be activated by BFA-insensitive guanine nucleotide-exchange proteins (27). In addition, BFA has actions that are apparently independent of ARF, such as stimulation of ADP-ribosylation (28), which further complicates the interpretation of effects observed in intact cells.

Although there is evidence that the same PLD might be activated by ARF, Rho, and/or PKC (19), some forms of PLD are clearly not regulated by PKC or guanine nucleotide-binding proteins. Several studies do support a role for ARF and Rho in PLD activation (29). For example, ARF and Rho acted synergistically in the activation of brain membrane PLD (7). Rho, ARF, and PKC were implicated in PLD activation in HL-60 (30) and brain cell membranes (7); clearly, the effects in disrupted and intact cells may be very different. To evaluate the role of Rho in intact cells, a novel Sindbis virus vector was used to induce expression of C3 and Rho proteins in A549 cells (human adenocarcinoma cells). From the observations reported here, it appears that a rate-limiting step in PLD stimulation by PMA, BK, and SPP in A549 cells involves RhoA but not a BFA-sensitive ARF pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**

Palmitic acid (9,10–3H) (30–60 Ci/mmol), used to label cellular phospholipids, was purchased from NEN Life Science Products, standard lipids were from Avanti Polar Lipids, solvents were from Fluka, and silica gel 60 plates for thin layer chromatography were from Merck. PMA, BK, SPP, BFA, and other chemicals were from Sigma. Mouse monoclonal antibodies against a synthetic peptide with RhoA sequence were from Santa Cruz Biotechnology, and monoclonal antibodies against Golgi 58-kDa protein were from Sigma. Rabbit antiserum raised against bacterially synthesized C3 was a generous gift of Dr. M. Popoff (Institut Pasteur, Paris, France). Secondary antibodies (goat anti-mouse and anti-rabbit immunoglobulin G1 conjugated with fluorescein and rhodamine-B, respectively) were from BIOSOURCE. Human lung carcinoma A549 cells were from the American Type Culture Collection (ATCC, Manassas, VA). All materials for cell culture were from Biofluid.

**Methods**

**Cell Culture**—A549 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (5 units/ml), streptomycin (5 μg/ml), and 2 mM glutamine.

**Transphosphatidylation Assay of PLD in Intact Cells**—PLD activity was assayed by measuring [3H]phosphatidylethanol ([3H]PtdEtOH) produced via PLD-catalyzed transphosphatidylation in cells previously labeled with [3H]palmitic acid (31). Cells were grown in medium containing bovine serum albumin, 1 mg/ml, for 24 h with [3H]palmitic acid (10 μCi/ml) added for the last 18 h. The medium was then aspirated and after washing, cells were incubated in fresh medium for 2 h. To initiate experiments, 1% ethanol was added 5 min before agonists. Incubations, at 37 °C, were terminated by washing monolayers twice with ice-cold PBS and immediately adding 1 ml of ice-cold methanol. Cells were collected by scraping and dishes were washed with an additional 1 ml of ice-cold methanol. Lipids were then extracted essentially as described by Bligh and Dyer (32) except that water was replaced with 1 M NaCl. The lower phase was dried under a stream of nitrogen, and the residue was dissolved in a small volume of chloroform/methanol (2:1). [3H]PtdEtOH was separated from other phospholipids by TLC on silica gel 60 plates with the organic phase of the solvent mixture, chloroform/methanol/acetate acid (90:10:10, v/v/v), and unlabeled PtdEtOH as a
Inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.3 mM iodoacetamide) containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, and protease inhibitors were added to Sindbis virus-infected and lysed by sonification twice for 15 s on ice in Buffer A (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 5% glycerol, 5% dimethyl sulfoxide, 50 mM NaF, 5 mM Na3VO4, 1 mM iodoacetamide, 1 mM leupeptin, 10 ng/ml aprotinin, and 100 μg/ml bacitracin). Cells were lysed by sonification for 15 s on ice in Buffer B (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, and protease inhibitors). Rabbit IgG. Proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).}

**TABLE I**

| Treatment of cells | PLD activity | C3 | Plus C3 |
|--------------------|--------------|----|--------|
| Infected            | No           | 0.20 ± 0.04 |       |
| Scrapped            | No           | 0.22 ± 0.01 |       |
| C3                  | No           | 0.40 ± 0.03* |       |
| Plus C3             | No           | 0.43 ± 0.08* | 0.47 ± 0.05* |

* Expression of C3 or Rho19N or C3 treatment significantly increased PLD activity (P < 0.05) by Student's t test.

**RESULTS**

Expression of Recombinant Proteins in Cells—Infection of A549 cells with dsSIN-C3 led to cell rounding, consistent with reported effects of microinjected C3 (37). Two hours after removal of virus, cell morphology had returned to normal (Fig. 1).

Intracellular C3 exoenzyme was detected as early as 2 h after infection with an MOI of 3 (Fig. 2A). Because expression was maximal with an MOI of 20, this condition was used for all other experiments. No reaction of anti-C3 antibodies with proteins in dsSIN:CAT-infected A549 cells was found, consistent with the specificity of these antibodies. The expression of C3 in A549 cells was accompanied by a loss of immunodetectable ~20-kDa RhoA (Fig. 2B), probably because, as had been suggested for rat1 fibroblasts (13), ADP-ribosylation of RhoA increases its susceptibility to degradation. A significant level of immunoreactive RhoA decreased in dsSIN-C3-infected A549 cells, consistent with the expression of C3 in A549 cells (Fig. 2B).

**Fig. 2. Expression of recombinant C3 exoenzyme, Rho19N, and CAT in A549 cells.** A, effect of MOI on C3 exoenzyme expression. A549 cells were incubated for 1 h with Sindbis virus encoding CAT or indicated amounts (MOI) of Sindbis virus encoding C. botulinum C3 exoenzyme (dsSIN:C3). Fresh virus-free medium was added to infected cells 2 h before they were harvested for Western analysis. Proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis in 16% gel. Rabbit antiserum raised against bacterially expressed C3 was used to detect C3 exoenzyme. B, effect of expression of dsSIN:C3 on endogenous RhoA. Proteins (20 μg) from cells infected with dsSIN-Rho19N were processed as described above, and RhoA was detected with the mouse monoclonal anti-RhoA antibody.

Plates at a density of 5–7 × 10⁵ cells/plate and labeled when indicated with [3H]galactosamine, were washed with PBS and diluted in PBS containing 1% cattle serum and 2% bovine serum albumin in PBS without Ca²⁺ and Mg²⁺. Cells were transfected in 100-mm Petri dishes, grown to confluence on 100-mm Petri dishes, washed twice with PBS and 2 ml of scraping buffer (114 mM KCl, 1 mM NaCl, 5.5 mM MgCl₂, 10 mM Tris-HCl). C3 transfection (5 μg/ml) or vehicle was added in 0.5 ml of scraping buffer and the cells were gently scraped, suspended in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum, and split among six-well dishes. After 6–7 h of incubation, we estimated ~70% viable cells. Cells were labeled with [3H]galactosamine (5 μCi/ml) in serum-free medium overnight and used for PLD assay as described above.

**Scrape Loading—**A549 cells, grown to confluence on 100-mm Petri dishes, were washed twice with PBS and 2 ml of scraping buffer (114 mM KCl, 1 mM NaCl, 5.5 mM MgCl₂, 10 mM Tris-HCl). C3 transfection (5 μg/ml) or vehicle was added in 0.5 ml of scraping buffer and the cells were gently scraped, suspended in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum, and split among six-well dishes. After 6–7 h of incubation, we estimated ~70% viable cells. Cells were labeled with [3H]galactosamine (5 μCi/ml) in serum-free medium overnight and used for PLD assay as described above.

**Western Blotting—**To evaluate protein expression from recombinant Sindbis virus, cultured cells were harvested at specified times after infection and lysed by sonification twice for 15 s on ice in Buffer A (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, and protease inhibitors (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.3 mM aprotonin, and leupeptin and pepstatin, each 10 μg/ml). Total cellular protein was quantified, and samples (20–50 μg) were subjected to SDS-polyacrylamide gel electrophoresis in 16% gels. Proteins were transferred to nitrocellulose membranes, which were incubated overnight in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20 (TTBS 0.1% Tween 20 in Tris-buffered (pH 7.6) saline)) and 0.2% I-block (Tropix, Bedford, MA). Hybridization with mouse monoclonal antibodies against RhoA or rabbit antiserum raised against bacterially synthesized RhoA (2 h room temperature) was followed by washing with TTBS and incubation with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. Proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).
Effects of Rho Proteins on Basal PLD Activity—The involvement of Rho in PLD activation was demonstrated by infecting A549 cells with recombinant Sindbis virus vectors to express mutant Rho protein or \textit{C. botulinum} C3 exoenzyme, an ADP-ribosyltransferase that inactivates Rho. Expression of either C3 exoenzyme or the dominant negative Rho19N in A549 cells by infection with dsSIN:C3 or dsSIN:Rho19N, respectively, resulted in a doubling of basal PLD activity (Table I). The increase was similar in A549 cells scrape-loaded in the presence of C3 transferase. The effect of C3 after scrape loading was less than that after Sindbis infection (~50% stimulation versus ~100%). Both experiments, however, suggest that Rho proteins play a role in the regulation of PLD activity in human epithelial cells and may have a negative effect on the enzyme activity, restraining or minimizing the level of PLD in unstimulated cells, perhaps by inhibiting a specific PLD or interfering with its activation.

Effects of Rho Proteins on Agonist-stimulated PLD Activity—As shown in Fig. 3A, expression of either dsSIN:C3 or dsSIN:Rho19N reduced but did not abolish PMA stimulation of PLD activity, indicating that Rho proteins are involved in a PKC-dependent pathway. In cells infected with dsSIN:C3, BK or SPP failed to increase PLD activity (Fig. 3B). BK plus SPP, however, induced an ~80% increase in PLD activity, suggesting that the combination of agonists was able to overcome, in part, the inhibitory effects of C3, perhaps acting through Rho that had not been ADP-ribosylated by the exoenzyme. In scrape-loaded cells, the effect of C3 in reducing PLD stimulation by agonists was less than it was in infected cells, albeit statistically significant; in cells expressing C3, PMA stimulation was reduced 25 ± 2% versus 38 ± 4% (n = 3), and BK plus SPP stimulation was reduced 20 ± 1.5% versus 40 ± 9% (n = 3). These data are consistent with the conclusion that for demonstration of C3 effects, viral infection was more effective than scrape loading.

Incomplete Modification of Endogenous RhoA by C3 Trans-ferase—To explore the possibility that the incomplete reduction of PLD stimulation by C3 was due to differences in the accessibility of intracellular pools of Rho, we quantified ADP-ribosylation of RhoA in cell lysates. First to prove that C3 trans-ferase expressed in dsSIN:C3 infected cells was enzymatically active, 100 ng of exogenous RhoA (His6-Rho) and [14C]NAD were added to cell lysates (Fig. 4). A clear band of 20 kDa was detected, indicating that the C3 trans-ferase was able to catalyze ADP-ribosylation of RhoA. Then to verify whether the intracellular pools of Rho were completely modified by the expressed C3 trans-ferase, we performed experiments using exogenous C3 exoenzyme. ADP-ribosylation of endogenous Rho was not detected when C3 exoenzyme was added at final concentration of 0.025 μg/ml to lysates of cells infected with dsSIN:CAT or dsSIN:C3. After addition of higher concentration of C3 (0.5 μg/ml) (Fig. 5), a single radiolabeled band, corresponding in migration to endogenous Rho, was detected. The band at ~20 kDa was more intense in lane 4 than in lane 2, consistent with the larger amount of the overexpressed dominant negative Rho19N. The presence of a band in lane 6, although perhaps somewhat weaker than that in lane 2, suggested that even when the expression of C3 seemed maximal, modification of the endogenous Rho was not complete. Similarly, some endogenous Rho was inaccessible to the C3 in dsSIN:C3-infected cells that were treated with agonists (Fig. 6).
PLD activity in cells expressing C3 exoenzyme. The extent of ADP-ribosylation of endogenous Rho in dsSIN:C3-infected cells varied from one experiment to another, but it was always statistically significant when data were quantified by densitometry (data not shown). Because of the presence of unmodified RhoA, indirect immunofluorescence was used to localize C3 exoenzyme and RhoA in A549 cells. In dsSIN:C3-infected cells, C3 transferase was concentrated in the Golgi compartment and widely distributed in the cytosol (Fig. 7, panel 4). Endogenous RhoA in unstimulated cells appeared predominantly in Golgi and in endoplasmic reticulum (Fig. 7, panels 5 and 6). Thus, indirect immunofluorescence indicated colocalization of C3 and endogenous RhoA in Golgi and endoplasmic reticulum compartments.

Effects of BFA on Basal, PMA-stimulated, and Receptor-stimulated PLD Activity—The effects of BFA on Golgi structure and PLD activity in A549 cells were evaluated. Incubation for 1 h with BFA (5 µg/ml) induced a drastic redistribution of the Golgi 58-kDa immunoreactivity (Fig. 8, panels 2 and 3). Only a slight effect on Golgi structure was detected in PMA-treated cells (panel 4). In similar experiments, BFA significantly decreased PMA-stimulated secretion of IL-6 (control, 435 ± 45 pg/ml; 1 µM PMA, 1414 ± 65 pg/ml; PMA plus BFA, 431 ± 10 pg/ml). Both these findings indicated that A549 cells were fully sensitive to the well known effects of BFA on Golgi structure and function. As shown in Table II, incubation of A549 cells with BFA (5 µg/ml) for 30 min or 3 h (data not shown) did not significantly affect basal PLD activity or the stimulation by PMA, BK, SPP, or BK plus SPP. Nor was a significant effect of BFA observed with submaximal concentrations of these agonists (5 nM BK or 30 nM SPP). Synergistic effects of Rho and ARF proteins on PLD activity have been reported (7, 29). Using C3 exoenzyme, we investigated whether the Rho-dependent PLD activity was modified by BFA treatment. As shown in Table II, incubation with BFA (5 µg/ml, 30 min) had no significant effect on C3-induced PLD activity (Table II). No significant effect of BFA was also seen in cells scraped-loaded in the presence of C3 and stimulated with PMA (3.4 ± 0.38 versus 3.8 ± 0.31, n = 3) or BK plus SPP (1.4 ± 0.25 versus 1.8 ± 0.12, n = 3).

FIG. 5. ADP-ribosylation of endogenous Rho in A549 cells. Samples (20 µg) of lysates from cells infected with dsSIN:CAT, dsSIN:Rho19N, or dsSIN:C3 were incubated with [32P]NAD with or without C3 exoenzyme (0.5 µg) and processed as described in Fig. 4. (A) rRhoA and recombinant proteins with minimal cytotoxicity (33). The cells, or does so only poorly, making it inconvenient to use in living cells to probe Rho function. Electroporation, permeabilization, prolonged incubation (days), and scrape loading procedures have been used to effect intracellular delivery of C3 (13, 15, 37). To avoid these manipulations and preserve fully responsive cells, in this study, A549 cells were infected with recombinant Sindbis virus encoding C3 exoenzyme (dsSIN:C3) or the dominant negative Rho19N (dsSIN:Rho19N). Infection of cells with these vectors resulted in a high level of expression of recombinant proteins with minimal cytotoxicity (33). The cells, PLD activity in human airway epithelial cells, cultured A549 cells were treated with PMA, a direct activator of PKC, and with agonists that act through G protein-coupled receptors, i.e. bradykinin (BK), a proinflammatory peptide shown to play a critical role in the development of airway hyper-responsiveness, and SPP, which has been implicated in several biological responses (38). Involvement of 20-kDa GTP-binding proteins in PMA- or receptor-mediated activation of PLD has been reported (19), although it seems clear that the pathways utilized can differ with different agonists and cell types.

C. botulinum exoenzyme C3 ADP-ribosylates Asn-41 in Rho, which is within the putative effector domain of the molecule (12). Because this covalent modification specifically inactivates Rho, C3 can be a useful probe of Rho function and has been used in A549 cells to investigate the role of the Rho GTPase in bradykinin-stimulated nuclear factor-κB activation and IL-1b gene expression (39). Unfortunately, C3 does not enter intact cells, or does so only poorly, making it inconvenient to use in living cells to probe Rho function. Electroporation, permeabilization, prolonged incubation (days), and scrape loading procedures have been used to effect intracellular delivery of C3 (13, 15, 37). To avoid these manipulations and preserve fully responsive cells, in this study, A549 cells were infected with recombinant Sindbis virus encoding C3 exoenzyme (dsSIN:C3) or the dominant negative Rho19N (dsSIN:Rho19N). Infection of cells with these vectors resulted in a high level of expression of recombinant proteins with minimal cytotoxicity (33). The cells,
2 h after removal of virus, demonstrated apparently normal Golgi localization of p58. Viral infection did not alter cell metabolism nonspecifically, at least in so far as was manifest by effects on incorporation of radiolabeled palmitic acid into phospholipids or on phosphatidylethanol formation (basal or agonist-stimulated). Moreover, lysates from infected cells were still able to ADP-ribosylate recombinant His6-Rho added \textit{in vitro}. When Sindbis infection was used in studies of basic cell functions, such as posttranslational processing (40, 41) or programmed cell death (36), no cytotoxicity was reported. We observed that cell viability was, in fact, higher than with scrape loading (70–80\% versus 60–70\%). A disadvantage of the Sindbis expression system, however, is that some cell types cannot be used for long term experiments (longer than 8 h) because virus replication inhibits host protein synthesis. To check for effects on cell metabolism, protein synthesis was examined by incubating cells with \textsuperscript{3}H]methionine for 3 h after removal of the virus; an approximately 10% inhibition of protein labeling was observed (data not shown). In A549 cells, expression of C3 exoenzyme was rapid and efficient as confirmed by Western analysis and changes in cell morphology during virus incubation.

RhoA is present in A549 cells at very low levels, but was detectable in homogenates after radiolabeling \textit{in vitro} with \textsuperscript{32}P]NAD and exogenous C3 exoenzyme. C3 expression was accompanied by a decrease in the amount of immunoreactive RhoA, as previously demonstrated in fibroblasts treated with C3 exoenzyme (13), probably because C3-catalyzed ADP-ribosylation accelerated its degradation. Loss of RhoA was observed under conditions in which not all of the RhoA had been modified by C3. As shown here, the loss of RhoA protein in cells expressing C3 was associated with PLD activation. Because a similar increase in activity was observed in cells expressing the dominant negative Rho19N, it was presumably not due to a nonspecific action of C3 on other types of Rho proteins. Moreover, the C3-induced increase in PLD activity was not due to an effect of the viral infection because a significant increase was also observed in cells scrape-loaded with C3. Together, these findings indicate that the regulation of PLD in A549 cells involves specifically RhoA and is the first evidence of negative regulation of PLD by RhoA in intact cells.

Inactivation of RhoA-dependent pathways with C3 or a dominant negative RhoA (Rho19N) enhanced basal PLD. The only prior reports of effects of inactivation of Rho on PLD activity in

**FIG. 7. Immunolocalization of C3 exoenzyme (A) and endogenous Rho (B).** Cells infected with dsSIN:C3 virus were fixed and double-labeled with mouse monoclonal antibodies against Golgi 58-kDa protein (panel 2) and rabbit antisem against C3 (panel 4), which were detected using anti-mouse fluorescein- and anti-rabbit rhodamine-B-conjugated IgG1, respectively. The two images are superimposed in panel 3. Monoclonal anti-RhoA and fluorescein-conjugated immunoglobulin IgG1 were used to detect endogenous RhoA in unstimulated A549 cells (panel 5). Nuclei (panel 6) were stained with 4’6-diamidino-2-phenylindole (blue). The coverslips were mounted on Mowiol (Hoescht) and examined with a Leica confocal microscope.
the absence of added effectors involved PC12 and HEK cells. Incubation of isolated membranes or permeabilized cells with the exoenzyme decreased or did not affect the basal activity, respectively (7, 17). Under conditions where basal PLD was increased, activation of PLD by PMA was reduced about 50% by infection of cells with dsSIN:C3 or dsSIN:Rho19N; PLD was not affected by infection with dsSIN:CAT, a recombinant virus encoding choramphenicol acetyltransferase, indicating that the cells were fully responsive after infection and consistent with a specific role for RhoA. Partial reduction of PMA-stimulated PLD by C3 has also been reported in rat1 fibroblasts (13).

We found that even when expression was optimal and all cells expressed C3 exoenzyme, a significant fraction of Rho protein was not ADP-ribosylated.

Although activation of PLD by ARF has been widely investigated in cell-free systems (3, 18, 19, 42), evidence for its occurrence in cells is limited (18, 20, 43). In many cells, processing and secretion of proteins are blocked by brief incubation with BFA, a fatty acid metabolite widely used to inhibit ARF action (44, 45). Natural resistance to BFA has been reported in PtK-1 and MDCK cell lines (46–48), and mutant CHO cells resistant to BFA have been isolated and characterized (49). The A549 cells used in the studies reported here were fully sensitive to effects of BFA on Golgi structure and function. Immunofluorescence microscopy using antibodies raised against the 58-kDa protein showed redistribution of this Golgi marker in cells treated for 1 h with BFA, 5 μg/ml. A significant decrease in IL-6 secretion was also observed. Similar treatment caused no significant alteration of PLD activity in A549 cells incubated with or without agonists, suggesting that BFA-sensitive guanine nucleotide-exchange proteins for ARF that are involved in the maintenance of Golgi structure and function do not directly influence or are not rate-limiting in the regulation of PLD activity. Similarly, Guillemin and Exton (26) recently reported that treatment of differentiated HL-60 cells with BFA for up to 6 h markedly altered the distribution of the trans-Golgi marker enzyme galactosyltransferase activity but did not affect PLD activation by formyl Met-Leu-Phe, ATP, or PMA. Because PLD activity was assayed in intact cells, inhibition of an ARF-dependent component by BFA treatment might be too small a fraction of the total to be detected. BFA also failed to affect PLD activity in cells expressing C3. Several explanations

### Table II

| Activators | Phospholipase D activity | No BFA | Plus BFA |
|------------|--------------------------|--------|----------|
|            | % [3H]PtdEtOH             |        |          |
| None       | 0.28 ± 0.04              | 0.44 ± 0.09 |
| C3 (5 μg/ml) | 0.47 ± 0.05          | 0.65 ± 0.08 |
| PMA, 1 μM  | 2.80 ± 0.17             | 2.40 ± 0.19 |
| BK, 5 nM   | 1.03 ± 0.20             | 0.82 ± 0.10 |
| BK, 1 μM   | 1.25 ± 0.13             | 1.01 ± 0.10 |
| SPP, 30 nM | 0.65 ± 0.04             | 0.61 ± 0.05 |
| SPP, 1 μM  | 1.20 ± 0.20             | 1.55 ± 0.15 |
| BK plus SPP| 1.90 ± 0.40             | 2.11 ± 0.38 |

* Significant effects on respective control (p < 0.05).
for the negative findings are possible, e.g. the effect of BFA on ARF activation may be incomplete so that sufficient active ARF remains to permit full PLD activation, but not secretion. It seems equally possible that activation of the ARF required for PLD activation may be catalyzed by a BFA-insensitive guanine nucleotide-exchange protein, such as cytohesin-1. Alternatively, PLD stimulation by active ARF in intact cells may be prevented by an inhibitor such as arfaptin (50). In any case, our observations are consistent with the notion that BFA-sensitive activation of ARF is not directly involved in the rapid stimulation of PLD induced by PMA, BK, or SPP, and also with the existence of a BFA-insensitive pathway that involves a PLD isof orm not required for IL-6 secretion.

Because inactivation of RhoA was accompanied by an increase of PLD activity, there appears to be a Rho-dependent inhibitory component(s) in A549 cells. This was not affected by BFA treatment, indicating that RhoA did not require BFA-sensitive ARF activation to regulate PLD activity. Both BFA treatment and Rho inactivation modify the cytoskeleton. Effects of C3 exoenzyme and Rho19N are not, however, simply due to a general disruption of signal transduction as a consequence of altered cytoskeletal organization or function, but to a more direct involvement of the Rho GTPase, which is consistent, at least, with the apparent contribution to PLD activation of a pool of Rho that is not completely accessible to C3 toxin. The data reported here do not exclude roles for ARF proteins in the regulation of PLD, but they indicate that a rate-limiting step in the regulation of basal and agonist-enhanced PLD activity in human A549 cells were dem-