Activation of Phospholipase D by Bradykinin and Sphingosine 1-Phosphate in A549 Human Lung Adenocarcinoma Cells via Different GTP-Binding Proteins and Protein Kinase C Delta Signaling Pathways†

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ABSTRACT: Phospholipase D (PLD) is involved in the signaling by many extracellular ligands, and its regulation appears to be quite complex. We investigated the signaling pathways initiated by bradykinin (BK) or sphingosine 1-phosphate (S1P) in A549 cells to define molecular mechanisms responsible for their additive effects on PLD activity. BK and S1P each elicited a sustained increase in phosphatidic acid content through a rapid and transient activation of PLD. The two pathways demonstrated rapid homologous downregulation, but heterologous desensitization was not observed. Action of both agonists required protein kinase C (PKC) activation and Ca^{2+} influx but was mediated by different heterotrimeric G proteins. In membranes, inhibition of PKCδ by rottlerin enhanced BK activation of PLD but inhibited that by S1P. Rottlerin inhibited activation of PLD in nuclei by both BK and S1P. By in situ immunofluorescence or cell fractionation followed by immunoblotting, PLD1 was concentrated primarily in nuclei, whereas the membrane fraction contained PLD2 and PLD1. Moreover, PKCδ specifically phosphorylated recombinant PLD2, but not PLD1. BK and S1P similarly enhanced RhoA translocation to nuclei, whereas BK was less efficacious than S1P on RhoA relocalization to membranes. Effects of both agonists on the nuclear fraction, which contains only PLD1, are compatible with a RhoA- and PKCδ-dependent process. In membranes, which contain both PLD1 and PLD2, the stimulatory effect of S1P on PLD activity can best be explained by RhoA- and PKCδ-dependent activation of PLD1; in contrast, the effects of BK on RhoA translocation and enhancement of BK-stimulated PLD activity by PKC inhibition are both consistent with PLD2 serving as its primary target.

Agonist-induced hydrolysis of specific phospholipids is crucial to many signaling pathways. In particular, phospholipase D (PLD)[1] activation has been implicated in the action of a number of hormones, growth factors, neurotransmitters, and cytokines (*1*−*3*) and in the control of important cell functions such as mitogenesis, vesicular trafficking, and cytoskeletal reorganization (*1*−*3*). Multiple PLD isoforms are involved in these regulatory actions. Two mammalian PLD isoenzymes, PLD1 and PLD2, specific for phosphatidylcholine (PtdCho), have been cloned (*4*, *5*). In addition, a phosphatidylethanolamine-specific PLD has been described (*6*).

PLD activity is under complex regulation that appears to be agonist- and cell-specific involving, in many instances, protein kinase C (PKC) and ∼20-kDa GTP-binding proteins, e.g., ARF, RhoA (*1*−*3*). A role for Ca^{2+} has also been suggested, although it remains a question whether the cation is required for Ca^{2+}-dependent PKCs or affects PLD through other signaling pathways. PLD2 was initially characterized as a constitutively active enzyme, insensitive to PLD activators such as PKCα, ARF, or RhoA, and regulated primarily by inhibitory factors residing in either the cytosol or cell membrane (*4*). In this regard, α-synuclein, *β*-synuclein, fodrin, and α-actinin were identified as in vitro inhibitors of PLD2 activity (*2*, *3*). Recent evidence, however, suggests that PLD2 activity is positively modulated by signaling pathways/effectors, which regulate PLD1 activity (e.g., ARF, PKCα) (*7*, *8*).

The mechanisms of PLD regulation by PKC are not well understood: it has been reported that PLD1 directly associated with PKCα, and phosphorylation by PKCα activated the enzyme in caveolin-enriched microdomains within the plasma membrane (*9*). Recently, in a human epithelial cell line, it was shown that PLD1 becomes phosphorylated via a PKCδ-dependent mechanism following challenge with S1P,

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[1] Abbreviations: PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdPro, phosphatidylpropanol; PtdEtOH, phosphatidylethanol; PLC, phospholipase C; PKC, protein kinase C; PTX, pertussis toxin; BK, bradykinin; S1P, sphingosine 1-phosphate; ARF, ADP-ribosylation factor; TLC, thin-layer chromatography; PARP, poly(ADP-ribose)polymerase; ODN, oligodeoxyribonucleotides.

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although evidence for direct interaction between the two proteins was not provided (*10*). In other cell systems, however, PLD1 appeared to be regulated by PKC through protein−protein interaction and not through phosphorylation (*11*, *12*). The regulation of PLD2 by PKC also appears to be controversial. It has been reported that PLD2 was phosphorylated on its Ser/Thr residues and phosphorylation resulted in inhibition of catalytic activity (*13*, *14*), suggesting that this isoform could be a negative effector of a Ser/Thr protein kinase. To the contrary, Han et al. (*15*) reported that PKCδ mediates the phosphorylation-dependent activation of PLD2 in PC12 cells.

Phosphatidic acid (PtdOH), produced by PLD-catalyzed hydrolysis of PtdCho (*1*−*3*), can function as a messenger and mediate some of the effects of PLD activation. Indeed, PtdOH activates important regulatory proteins in vitro [e.g., Raf-1 kinase, phosphatidylinositol 4-phosphate 5-kinase, phospholipase C (PLC)-γ (reviewed in ref *16*)] including a novel phosphatidate-dependent protein kinase (*17*). Of note, a direct interaction with PtdOH is responsible for agonist-dependent Raf-1 translocation (*18*) and mTOR activation in mitogenic signaling (*19*).

Previous studies had implicated PLD in signaling by the proinflammatory peptide bradykinin (BK) via mechanisms that involve Ca^{2+} influx, PKC activation, and the monomeric GTPase RhoA (*15*, *20*−*23*). Several reports have documented the ability of sphingosine 1-phosphate (S1P) to initiate PLD signaling (*23*−*26*), but its mechanism of action has not been clearly defined. In this regard, stimulation of PLD by S1P may be dependent on internalization of the bioactive lipid and its subsequent action as intracellular mediator (*27*); in other instances, however, S1P stimulation of PLD activity was triggered by interaction of the lipid with its specific receptors (*25*, *26*). In skeletal muscle cells, S1P activation of PLD was mediated by multiple regulators [e.g., PKCα, PKCδ, Rho, Ca^{2+} (*25*, *28*, *29*)], whereas in a human epithelial cell line, S1P action was dependent exsclusively on PKCδ (*10*).

We had reported that, in human lung adenocarcinoma A549 cells, BK and S1P stimulate PLD activity in an additive manner through mechanisms that involve, in both cases, RhoA activation (*23*).

We then investigated the signaling pathways triggered by BK or S1P to define the molecular mechanisms responsible for their additive effects on PLD activity. The effects of BK and S1P appear to be mediated by, respectively, pertussis toxin (PTX)-insensitive and -sensitive pathways, which are notable for their rapid downregulation and the absence of heterologous desensitization. For both agonists, Ca^{2+}- and PKC-dependent pathways are involved in PLD activation. However, the preferential localization of PLD1 in the nuclear fraction, the different role of PKCδ in PLD isoform regulation and the differences in translocation of RhoA to membrane and nuclear fractions in response to BK and S1P, support the view that, in A549 cells, S1P activates primarily PLD1 via RhoA and PKCδ, whereas BK activates PLD1 but inhibits PLD2 via a PKCδ-dependent pathway.

EXPERIMENTAL PROCEDURES

Materials. Biochemicals, cell culture reagents, BK, nifedipine, human recombinant PKCδ, phospholipids, and diacylglycerol were purchased from Sigma (Milan, Italy). Dulbecco's Minimal Essential Medium (DMEM) was obtained from Life Technologies (Paisley, U.K.). S1P, GF 109203X, and rottlerin were purchased from Calbiochem (La Jolla, CA). PTX was from List Biological Laboratories (Campbell, CA). Antisense phosphothioate oligodeoxyribonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Effectene transfection reagent was from Qiagen (Valencia, CA). Recombinant baculoviruses harboring cDNA encoding for human PLD1 or mouse PLD2 were a generous gift of Prof. M. Frohman (Stony Brook University, Stony Brook, NY). Antibodies against PKCδ and calnexin were from Transduction Laboratories (Lexington, KY). PLD1 and PLD2 anti-peptide antibodies were generated as described (*30*−*32*). Rat monoclonal anti-mouse PLD2 antibodies and recombinant Flag-PLD2 were kindly provided by Dr. Y. Kanaho (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Mouse monoclonal anti-RhoA and anti-p58 antibodies, rabbit polyclonal anti-poly(ADP-ribose)polymerase, and secondary antibodies (goat anti-rat or anti-mouse or anti-rabbit immunoglobulin G1 conjugated to horseradish peroxidase) for Western analysis were from Santa Cruz (Santa Cruz, CA). Secondary antibodies (goat anti-rat or anti-rabbit immunoglobulin G1 conjugated, respectively, with fluorescein or rhodamine-B) were from BioSource (Camarillo, CA). Organic solvents and Silica Gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). [2-^{3}H]-Glycerol (14.2 Ci/mmol), L-α-dipalmitoyl-[choline-methyl-^{3}H]phosphatidylcholine (50 μCi/mmol) and [γ-^{32}P]ATP (800Ci/mol) were purchased from New England Nuclear (Dreiech, Germany). Standard lipids were from Avanti Polar Lipids (Alabaster, AL).

Cell Culture. Human lung adenocarcinoma A549 cells (ATCC, Manassas, VA) were routinely grown in DMEM containing 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. For radiolabeling studies, cells were grown to confluence in six-well 35-mm plates, medium was changed to DMEM containing 0.1% BSA, and cells were labeled as described.

Cell Fractionation. Serum-starved A549 cells were incubated with or without 1 μM S1P or 1 μM BK for 30 s prior to Western analysis, whereas in PLD experiments, 2% ethanol was added to the medium, and the cells incubated for 10 min. Medium was then removed and cells were washed twice with ice-cold PBS, scraped in buffer A (50 mM Tris/HCl, pH 7.5, 10 mM NaCl, 1 mM KCl, 2 mM $MgCl_2$) containing 300 mM sucrose and protease inhibitors (1 mM AEBSF, 0.3 μM aprotinin, leupeptin, 10 μg/mL and pepstatin A, 10 μg/mL), and homogenized (Dounce, 80 strokes). Lysates were centrifuged (10 min, 750*g*). Particulate fraction, including heavy and light membrane fractions, was prepared from the supernatant by centrifugation at 200000*g* for 1 h. Nuclei were prepared in three successive steps: the cell pellet from the centrifugation at 750*g* was gently dispersed in buffer A containing 300 mM sucrose and 0.5% Triton X-100, incubated at 4 °C overnight, and centrifuged (750*g*, 10 min). Use of the nonionic detergent Triton X-100 eliminates the outer nuclear membrane, leaving the morphology of nuclei similar to that of nuclei in intact cells. For further purification by sucrose gradient centrifugation, a sample of the low-speed pellet suspended in buffer A (100

μL) containing 300 mM sucrose was layered over 1.5 mL of 45% sucrose in 10 mM Hepes, pH 7.4, 1 mM EGTA, 1 mM EDTA, and centrifuged (2000*g*, 30 min) to separate adherent membranes from the nuclei (*33*). The pellet was dispersed in buffer A containing 2.2 M sucrose and centrifuged (50000*g*, 1 h). The supernatant was discarded, and the pellet of purified nuclei suspended in buffer A was used for Western analysis and activity assay.

Contamination of the nuclear fraction with endoplasmic reticulum was evaluated by measuring α-glucosidase type II activity according to Little et al. (*34*). Briefly, extracts were incubated with 1 mM 4-methylumbelliferyl α-D-glucoside in 100 mM sodium phosphate, pH 7.0/0.1% Triton X-100 for 15 min at 37 °C, quenched with 10 vol of ice-cold 250 mM potassium glycine, pH 10.3, and released 4-methylumbelliferone was measured using a Shimadzu RF5000 spectrophotofluorimeter (Ex 360 nm, Em 446 nm). α-Glucosidase activity per milligram of protein in nuclei was $8.0 \pm 1.1\%$ ($n = 3$) of that in endoplasmic reticulum. Purity of nuclear fraction was also assessed by Western analysis using antibodies against calnexin, a specific marker of endoplasmic reticulum, and poly(ADP-ribose)polymerase (PARP), a specific nuclear marker.

Measurement of [^{3}H]PtdOH Levels. Confluent cells were incubated with [^{3}H]glycerol (5 μCi/mL) for 24 h and then with agonist for the indicated time. Lipids were extracted essentially as described by Bligh and Dyer (*35*) except that 1 M NaCl replaced water. [^{3}H]PtdOH was isolated by TLC with chloroform/methanol/acetic acid (9:1:1). Positions of lipids were compared with those of authentic standards after staining with iodine vapor.

Measurement of [^{3}H]Phosphatidyl Alcohol Formation in Cells. [^{3}H]Phosphatidylpropanol (PtdPro) or [^{3}H]phosphatidylethanol (PtdEtOH) formation was chosen as measure of PLD activity since the enzyme can catalyze a transphosphatidylation reaction which preferentially utilizes primary alcohols instead of water. Confluent cells had been incubated for 24 h with [^{3}H]glycerol (5 μCi/mL) followed by stimulation in the presence of 2% 1-propanol or ethanol. Incubations were performed at 37 °C and terminated by washing cells twice with ice-cold PBS, followed immediately by addition of 1 mL of ice-cold methanol. For determination of total PLD activity in lysates, cells were scraped in ice-cold methanol; for quantification of [^{3}H]phosphatidyl alcohol in subcellular fractions, cells were scraped in homogenization buffer and differential centrifugation of homogenates was performed as described above. Lipids were then extracted and [^{3}H]PtdPro or [^{3}H]PtdEtOH was separated by TLC and quantified as described (*23*).

Indirect Immunofluorescence. For indirect immunofluorescence experiments, A549 cells grown on sterile glass coverslips or purified nuclei were fixed in 2% paraformaldehyde in PBS for 30 min at room temperature before permeabilization by a 30 min incubation with 0.05% saponin and 1% BSA, in PBS without Ca^{2+} and Mg^{2+}. Cells fixed on coverslips were incubated overnight with polyclonal antibodies against PLD1 (antiserum 4 and 41), monoclonal antibodies against PLD2 or polyclonal antibodies against PLD2 (antiserum 42), washed four times, and incubated for 1 h with goat anti-rabbit fluorescein- or rhodamine B-conjugated immunoglobulin G (IgG). After washing in PBS containing 0.05% saponin and 1% BSA, coverslips were mounted on Mowoil (Aventis, Strasbourg, France), viewed with a Nikon confocal microscope equipped with appropriate filters, and scanned with SCANware program.

Western Blot Analysis. Cells grown in 100-mm dishes, incubated without or with agonists, were washed in PBS, scraped in buffer A, and processed as described for "Cellular Fractionation". Samples in Laemmli's SDS-sample buffer were applied to polyacrylamide gels and subjected to electrophoresis for 90 min at 100 mA, before transfer of proteins to nitrocellulose membranes, which were incubated overnight at room temperature with 20 mM Tris, pH 7.5, 150 mM NaCl containing 0.1% Tween 20 and 0.5% I-block (Tropix, Bedford, MA). After rinsing, membranes were incubated for 1 h with antibodies against PLD1, PLD2, PKCδ, RhoA, calnexin, or PARP. Bound antibody was detected using chemiluminescence reagents (ECL, Amersham, Milan, Italy).

Oligodeoxyribonucleotide Administration. For inhibition of protein expression, oligodeoxyribonucleotides (ODN) were designed with nonconserved, nonrepetitive sequence corresponding to the region of translation initiation of PKCδ (5′-gcgccatggtggggcctgca-3′). As a control, a scrambled ODN (5′-ttttccgaggtagtaccgtg-3′) was used. Cells were loaded with ODN (2 μM) using Effectene transfection reagent, medium was replaced, and cells were incubated for 32 h, before processing for PLD assay as described above. The effect of ODN on PKCδ protein was evaluated by quantitative immunoblot analysis.

Phosphorylation of PLD Isoforms by PKCδ in Vitro. Human recombinant PKCδ was used for in vitro phosphorylation of PLD isoforms (*36*). Sf9 cells were infected with the recombinant baculoviruses harboring the cDNA encoding for human PLD1 or mouse PLD2 and propagated as described (*4*, *5*). Lysates of Sf9 cells overexpressing PLD1 or PLD2 or lysates of COS7 cells overexpressing mouse Flag-PLD2 were incubated (30 °C, 20 min) in 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 10 mM MgATP, 2 μCi ATP[γ^{32}P] (800 Ci/mol), 200 μg/mL phosphatidylserine, 20 μg/mL diacylglycerol, 0.03% Triton X-100, and 0.5 μg of human recombinant PKCδ without or with rottlerin. Reaction was stopped by adding trichloroacetic acid (final concentration 25%) After 16 h at −20 °C, proteins were collected by centrifugation (14.000*g*, 5 min), dissolved in Laemmli buffer, and separated by SDS−PAGE; the gel was dried and exposed to Kodak film.

To assess the effect of PKCδ-catalyzed phosphorylation on PLD2 activity, lysates of Sf9 cells overexpressing PLD2 were incubated as described above, except that unlabeled ATP was used. Samples (0.5 μg of protein) were utilized for PLD activity in vitro assay according to Brown et al. (*37*). Briefly, lysates were mixed on ice in 50 μL of 50 mM Na-HEPES, pH 7.5, 3 mM EGTA, 80 mM KCl, 1 mM DTT, 3 mM $MgCl_2$, and 2 mM $CaCl_2$. The substrate was added in the form of phospholipid vesicles composed of phosphatidylethanolamine, phosphatidylinositol 4,5-bisphosphate, and dipalmitoyl-phosphatidylcholine in a molar ratio of 16:1.4:1, respectively. L-α-Dipalmitoyl-[choline-methyl-^{3}H]-phosphatidylcholine was included to give about 50 000 cpm/assay. Vesicles (10 μL) were added to the samples (final concentration of PtdCho was 8.6 μM), and activity was measured at 37 °C for 15 min. Reactions were stopped by the addition of 200 μL of 10% trichloroacetic acid and 100

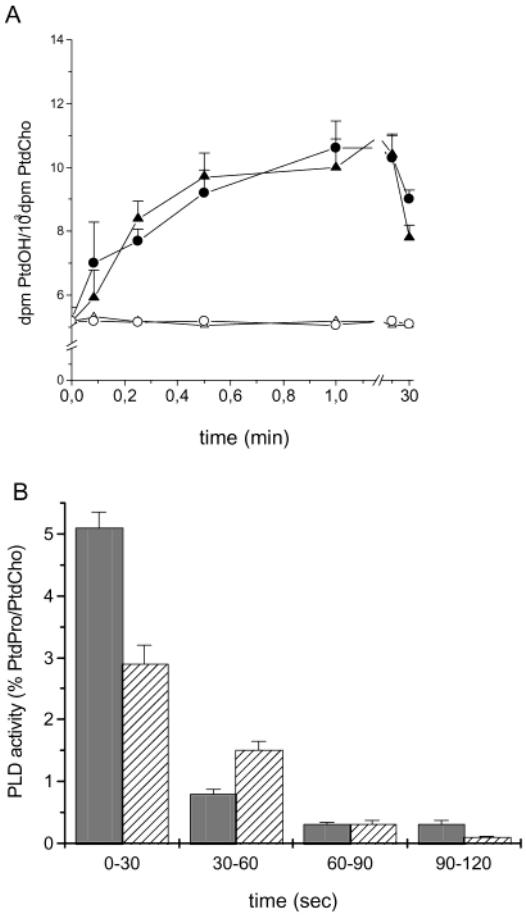

FIGURE 1: Time-course of the effects of BK and S1P on [^{3}H]PtdOH levels and PLD activity in A549 cells. (A) [^{3}H]Glycerol-labeled cells (5×10^5) were incubated for the indicated time with 1 μM BK (●, ○) or 1 μM S1P (▲, △) without (●, ▲) or with (○, △) 2% 1-propanol. Data are means ± half the range of values from duplicate assays in one experiment representative of three. Increase in [^{3}H]PtdOH levels induced by BK or S1P after 15 s of stimulation was significantly different by *t*-test from controls ($p < 0.05$). Where not shown, error bars lie within the symbol. (B) [^{3}H]Glycerol-labeled cells (5×10^5) were incubated with 1 μM BK (filled bar) or 1 μM S1P (hatched bar). 1-Propanol (2%) was present during the indicated 30 s interval to measure PLD activity during that period after addition of agonist. Lipids were then extracted and subjected to TLC as described in the Experimental Procedures. [^{3}H]-PtdPro formed is reported as percentage of total labeled PtdCho. Data are means ± SEM of values from triplicate assays in one experiment representative of at least three.

μL of 10 mg/mL bovine serum albumin. Precipitated lipids and protein were sedimented by centrifugation (3000*g*, 10 min, 4 °C). Sample of the supernatant (0.3 mL) was removed and analyzed by liquid scintillation spectroscopy.

RESULTS

Effect of BK or S1P on Activation of PLD and [^{3}H]PtdOH Generation in A549 Cells. In A549 cells, BK or S1P induced a rapid and sustained 2-fold increase in radiolabeled PtdOH, which reached a maximum within 1 min, persisted with little change for 15 min, and was still significantly higher than basal at 30 min. [^{3}H]PtdOH generation was prevented by incubation of the cells with 2% 1-propanol, which is preferentially utilized instead of water by PLD in a trans-phosphatidylation reaction (Figure 1A). PtdOH levels in agonist-stimulated cells were unaffected by the diacylglycerol kinase inhibitor R59949 or phosphatidate phosphohydrolase inhibitor propranolol, indicating that these enzymatic activities did not contribute significantly to PtdOH levels in A549 cells (data not shown). Hence, the effects of both BK and S1P are attributable to agonist-induced PLD activity. To examine the time course of PLD activation, 2% 1-propanol was added for 30 s periods (Figure 1B). Both agonists induced rapid and transient enzyme activation, which was maximal within the first 30 s, declined thereafter, and was almost undetectable after 60 s. Therefore, the rapid agonist-induced activation of PLD followed rapidly by its deactivation produced a prolonged increase in PtdOH levels. Both BK and S1P activated PLD in a concentration-dependent manner with half-maximal effects induced by 3 nM BK or 12 nM S1P (data not shown).

Table 1: PLD Activation by BK and S1P in A549 Cells: Homologous Desensitization and Absence of Cross-Desensitization[a]

additions		PLD activity
first	second	[^{3}H]PtdPro/[^{3}H]PtdCho
-	-	0.21 ± 0.03
BK	-	1.18 ± 0.05
BK	BK	1.26 ± 0.08
BK	S1P	1.84 ± 0.05*
S1P	-	0.88 ± 0.08
S1P	S1P	0.98 ± 0.09
S1P	BK	1.90 ± 0.08*

[a] Cells were incubated for 2 min with 1 μM BK or 1 μM S1P (first addition) in the presence of 2% 1-propanol and washed twice with PBS. Medium containing 2% 1-propanol was added and cells were incubated for further 2 min without or with the second addition. Data are means ± SEM from one experiment representative of at least three. (*) Significantly different from the stimulation of BK or S1P alone (Student's *t*-test; $p < 0.05$).

Absence of Cross-Desensitization of BK- and S1P-Induction of PLD Activation. In a previous study aimed at dissecting the role of monomeric GTP-binding proteins in agonist-stimulated PLD activity in A549 cells, BK and S1P stimulated PLD activity in an additive manner (*23*). To understand the molecular basis of the observed additivity, homologous and heterologous desensitization experiments were performed utilizing 1 μM BK and 1 μM S1P capable to elicit the maximal effect (Table 1). A second addition of S1P did not further increase [^{3}H]PtdPro in cells previously exposed to S1P for 2 min. Similar homologous desensitization was observed with BK. Cells previously stimulated with S1P or BK, however, were responsive to subsequent treatment with BK or S1P, respectively (Table 1), indicating an absence of cross-desensitization, consistent with the additive effects on PLD activity. Similarly, when [^{3}H]PtdOH levels were measured, homologous, but not heterologous desensitization, to the two agonists, was observed, excluding the possibility that PtdOH accumulation per se resulted in PLD inhibition (data not shown).

Involvement of Heterotrimeric G Proteins, PKC, and Ca^{2+} in Agonist-Induced PLD Activation. Incubation of cells with PTX (100 ng/mL) for 20 h abolished subsequent activation of PLD by S1P (Figure 2), consistent with a receptor-mediated action of the bioactive lipid, involving heterotrimeric GTP-binding proteins of the Gi/Go family. Toxin treatment did not, however, affect BK action, consistent with PLD activation via a PTX-insensitive type of heterotrimeric G protein (e.g., Gq).

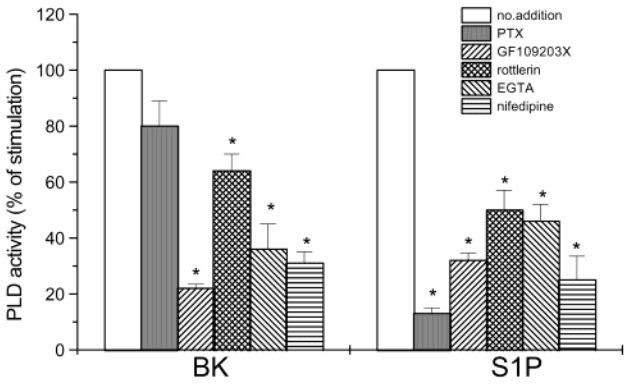

FIGURE 2: Effect of inhibition of Gi/Go, Ca^{2+} chelation, and inhibition of PKC on PLD activation by BK and S1P. [^{3}H]glycerol-labeled cells (5×10^5) were incubated for 20 h with 100 ng/mL PTX, for 30 min with 10 μM GF 109203X or 5 μM rottlerin, for 5 min with 2.5 mM EGTA, or for 10 min with 100 μM nifedipine. 1-propanol (2%) was then added for 5 min followed by incubation with 1 μM BK or 1 μM S1P for 10 min. The inhibitors had no effect on basal incorporation of radioactivity into [^{3}H]PtdPro in the absence of agonists. Data are means ± SEM of at least three independent experiments performed in duplicate. Effects of potential inhibitors on activation of PLD by BK or S1P were significant [(*) $p < 0.05$ by *t*-test]. PLD activation is reported relative to that produced by BK or S1P in the absence of potential inhibitors = 100%.

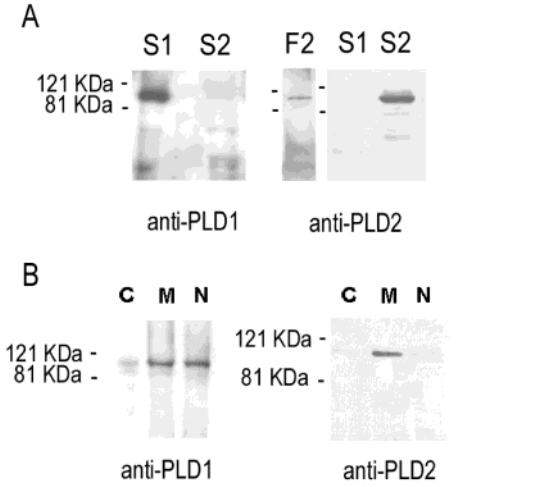

FIGURE 3: Subcellular localization by Western analysis of PLD1 and PLD2. (A) Proteins (20 μg) in lysates of Sf9 cells over-expressing PLD1 (S1) or PLD2 (S2) or mammalian cells over-expressing Flag-PLD2 (F2) were separated by SDS−PAGE, electroblotted to nitrocellulose, and hybridized with rabbit polyclonal anti-PLD1 or rat monoclonal anti-mouse PLD2 antibodies. (B) Proteins (25 μg) in cytosol (C), total membrane (M), and nuclear (N) fractions from A549 cells were subjected to Western analysis as in panel A using polyclonal anti-PLD1 and monoclonal anti-PLD2 antibodies as described in the Experimental Procedures. Data are from one experiment representative of four.

The stimulatory action of BK or S1P on PLD activity was significantly reduced by 10 μM GF 109203X, an inhibitor of classical and novel PKC isoforms (Figure 2). Rottlerin, a bisindolylmaleimide kinase inhibitor, selective for PKCδ, also reduced, although to a lesser extent, the effects of both agonists, suggesting a role for the Ca^{2+}-independent PKC isoform in the regulation of PLD activity in A549 cells (Figure 2).

Similarly, addition of EGTA to the medium or treatment with nifedipine, a Ca^{2+} channel blocker, markedly decreased the effects of BK and S1P, indicating that influx of extracellular Ca^{2+} was needed for the activation of PLD by S1P or BK (Figure 2). Moreover, in cells incubated for 15 s with BK or 30 s with S1P, a significant accumulation of inositol phosphates was observed, consistent with activation of PLC and generation of diacylglycerol, a physiological PKC activator (data not shown). Thus, a Ca^{2+}-dependent step, which likely involves the activity of conventional isoforms of PKC, was required for BK and S1P enhancement of PLD activity.

PLD Isoform Immunolocalization. Given that two different isoforms, PLD1 and PLD2, may account for PLD activity, subcellular localization of endogenous proteins in A549 cells was examined by Western analysis and indirect immunofluorescence. First, the specificity of polyclonal anti-PLD1 and anti-PLD2 antibodies and rat monoclonal anti-mouse PLD2 antibodies was tested using lysates from Sf9 cells or mammalian cells overexpressing PLD isoforms. As shown in Figure 3A, polyclonal anti-PLD1 and monoclonal anti-PLD2 antibodies were able to detect the PLD isoform with high specificity. Analogous results were obtained using polyclonal anti-PLD2 antibodies (data not shown). Western analysis of PLD1 and PLD2 in A549 cell fractions showed that PLD1 was clearly detectable in membrane and nuclear fractions, while it was less evident in cytosolic fraction; PLD2 was instead predominantly found in membrane fraction, but only slightly in cytosol and nuclear fractions (Figure 3B). These results were confirmed by indirect immunofluorescence. PLD1 was detected primarily in nuclei whereas PLD2 was found diffusely distributed in intracellular districts, but not in nuclei. PLD2 isoform partially colocalized with Golgi apparatus as demonstrated by the merging with the specific protein marker p58 whereas PLD1 localization in Golgi was less evident (Figure 4, right panel).

Subcellular Distribution of [^{3}H]PtdEtOH Generated by PLD Activity. To characterize further whether additive effects of BK and S1P on PLD were detectable in subcellular fractions, A549 cells were labeled with [^{3}H]glycerol and incubated with ethanol without or with agonist followed by cell membrane fractionation. Purity of membrane and nuclear fractions was tested by Western analysis. As shown in Figure 5A, PARP, a marker for nuclei, was present only in the nuclear fraction, whereas calnexin, a specific marker for endoplamic reticulum, was detected only in total membrane fractions. The α-glucosidase activity typically present in endoplasmic reticulum was also measured in both subcellular fractions (Figure 5A): the low α-glucosidase activity in nuclei indicated a high level of purity of this fraction. In the same figure the subcellular distribution of basal [^{3}H]PtdEtOH formation is shown. Approximately one-fourth of the total labeled-phosphatidyl alcohol was associated with the nuclear fraction, whereas a lesser amount of phosphatidyl alcohol was associated with the cytosolic fraction. As illustrated in Figure 5B, S1P and BK significantly affected PLD activity in cytosol, membrane, and nuclear fractions. Moreover, the effects of both together were greater than that of either alone in each fraction, but possibly not strictly additive, for reasons that are unclear. A possible explanation for this finding is the preferential activation by one or both agonists of PLD isoforms with different subcellular distributions.

Effect of BK or S1P on RhoA Translocation. Participation of RhoA in PLD activation by BK and S1P has been

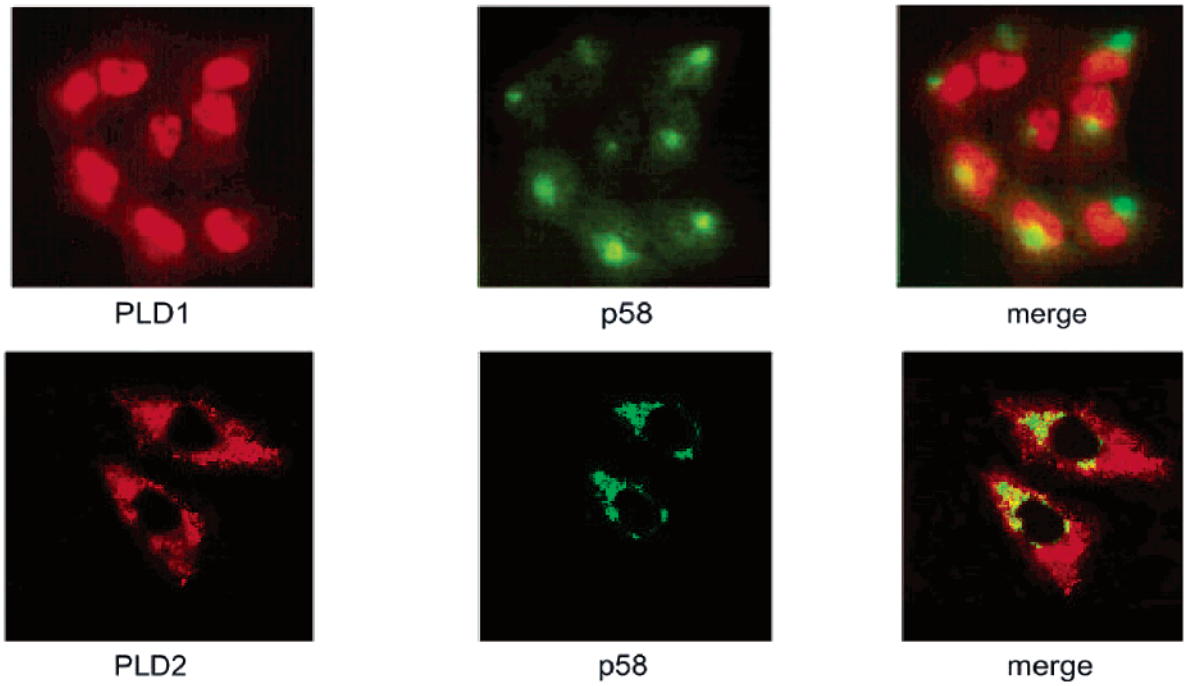

FIGURE 4: Immunolocalization of endogenous PLD1 and PLD2 in A549 cells. A549 cells grown on coverslips were fixed and processed for immunofluorescent staining using polyclonal anti-PLD1 or monoclonal anti-PLD2 as primary antibodies followed by rhodamine-B- or fluorescein-conjugated anti-rabbit or anti-rat IgG1, respectively. Coverslips were mounted on Mowoil and examined with a Nikon confocal microscope. Experiment shown is representative of four.

demonstrated (*23*). To investigate this point further, we examined RhoA translocation within cells following incubation with agonists for 30 s. As shown in Figure 6, the amount of RhoA present in membrane fractions was markedly increased by S1P and to a lesser extent by BK, whereas the amount present in nuclear fractions was increased similarly by both agonists.

Role of PKCδ in PLD Regulation by BK and S1P in Different Subcellular Fractions. Recently, PLD2 regulation by BK through a PKCδ-dependent pathway in PC12 cells was reported (*15*). To understand better the molecular mechanisms responsible for additive stimulation of PLD by BK and S1P, the role of PKCδ was investigated further. Treatment of A549 cells with S1P or BK for 30 s significantly increased translocation of PKCδ to membranes, indicating that both agonists activated PKCδ (data not shown). PLD1 and PLD2 were tested as possible substrates for PKCδ. In in vitro experiments, recombinant PLD2 synthesized in Sf9 cells was phosphorylated by PKCδ, which did not modify recombinant PLD1. The PKCδ-catalyzed phosphorylation of recombinant PLD2 did not affect PLD activity in vitro assay (85 ± 12 pmol versus 100 ± 22 pmol choline released in control and PKCδ-incubated lysates, respectively). Moreover, addition of rottlerin prevented the phosphorylation of PLD2 by PKCδ (Figure 7). Rottlerin also reduced the accumulation of PtdEtOH in the nuclear fraction from A549 cells induced by both agonists (Figure 7), similar to the effect observed in whole lysates (Figure 2), suggesting the involvement of PKCδ in the regulation of PLD1, which is the PLD isoform exclusively represented in nuclei. Surprisingly, opposite effects of PKCδ on BK- and S1P-stimulated PLD activity in total membranes were observed. Indeed, while rottlerin significantly decreased the effect of S1P, it greatly enhanced stimulation of PLD by BK.

To provide further evidence of the different roles of PKCδ in the regulation of PLD activity by BK in nuclear and total membrane fractions, the antisense RNA technology was used. Figure 8 (inset) shows that cell treatment with antisense PKCδ ODN significantly reduced PKCδ expression. The specificity of ODN for PKCδ was evaluated from immunoblot analysis of PKCα, which was already implicated in PLD regulation in other cell systems (*8*, *9*, *25*) (data not shown). Results obtained in cells in which PKCδ was downregulated confirmed previous findings obtained in the presence of rottlerin: total membrane-associated PLD activity stimulated by BK was enhanced, whereas nuclear-associated BK-stimulated PLD1 activity was markedly decreased (Figure 8). Since membrane-associated PLD activity includes both PLD2 and PLD1 and BK-stimulated PLD1 is inhibited by rottlerin, it seems likely that rottlerin enhances the BK effect on PLD2.

DISCUSSION

In the studies reported here, signaling pathways leading to PLD activation by BK and S1P in A549 lung epithelial cells were investigated. Additive stimulation of PLD by the two agonists was consistent with the involvement of different pathways triggered by BK and S1P through interaction with their specific receptors coupled, respectively, to PTX-insensitive and -sensitive heterotrimeric GTP-binding proteins. These findings are in agreement with our previous observation in skeletal muscle cells (*25*), a study performed in CFNPE9o$^-$ epithelial cells (*26*), and, in part, with findings in NIH3T3 fibroblasts (*38*), where, unlike our findings, S1P activated PLD via a G protein(s) distinct from Gi. The notion that BK and S1P activate PLD through separate signaling pathways is further supported by the demonstrated absence of cross-desensitization by the two agonists. Despite signaling

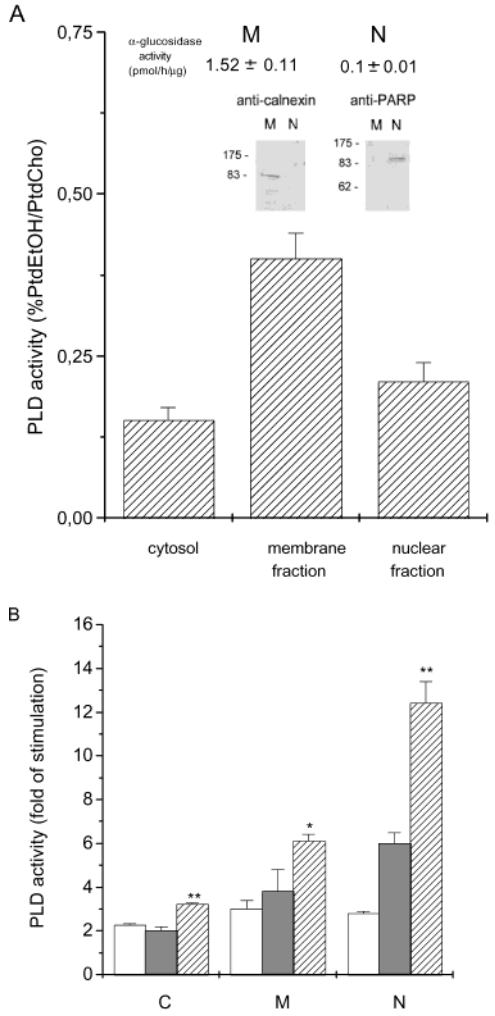

FIGURE 5: Subcellular distribution of [^{3}H]PtdEtOH generated by PLD activity. (A) Cytosolic, total membrane, and nuclear fractions prepared from [^{3}H]glycerol-labeled A549 cells were assayed for PLD activity as described in Experimental Procedures. Data are means ± SEM of values from six independent experiments performed in duplicate. (Inset) Samples (20 μg) of total membrane (M) or nuclear (N) fractions from S1P-treated cells were analyzed for calnexin and PARP immunoreactivity by immunoblotting. The same fractions were analyzed for the presence of α-glucosidase activity. Data are representative of three experiments. (B) Cytosolic (C), total membrane (M), and nuclear (N) fractions prepared from A549 cells incubated for 10 min with 1 μM BK (filled bar), 1 μM S1P (empty bar), or BK plus S1P (hatched bar) were assayed for PLD activity as described in the Experimental Procedures. Data are means ± SEM of values from duplicate assays in one experiment representative of at least three. Significantly different from the stimulation of BK or S1P alone. [Student's *t*-test; (*) $p < 0.05$; (**) $p < 0.005$].

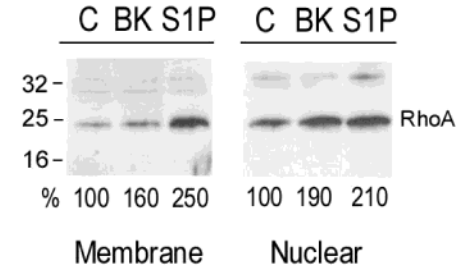

FIGURE 6: Effect of BK or S1P on RhoA translocation. Proteins (20 μg) in total membrane and nuclear fractions from A549 cells incubated without or with 1 μM BK or 1 μM S1P were subjected to Western analysis for RhoA, using mouse monoclonal anti-RhoA antibodies for immunodetection. Data are from one experiment representative of three. Band intensity is reported as percentage (%) relative to control (no addition, 100%; standard deviation was less than 15%).

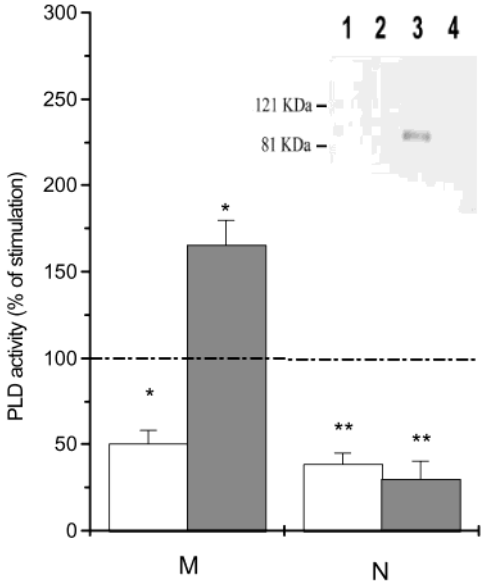

FIGURE 7: Effects of rottlerin on phosphorylation of PLD1 or PLD2 by PKCδ and on activation of PLD by BK and S1P in subcellular fractions of A549 cells. Total membrane (M) and nuclear (N) fractions prepared from A549 cells incubated for 30 min with 5 μM rottlerin and stimulated with 1 μM BK (■) or 1 μM S1P (□) were assayed for PLD activity. Activities are reported as the increase produced by each agonist in the presence of rottlerin expressed relative to that produced by the same agonist in its absence = 100%. Data are means ± half the range of values from duplicate assays in one experiment representative of at least three. In the absence of rottlerin, BK stimulation was 127% of that by S1P. Dashed line indicates those values set to equal 100% for each. All effects of rottlerin were statistically significant [(*) $p < 0.05$; (**) $p < 0.01$ by *t*-test]. (Inset) Proteins (20 μg) in lysates of Sf9 cells over-expressing PLD1 (1, 2) or PLD2 (3, 4) were incubated for 20 min at 30 °C with 0.5 μg of recombinant human PKCδ and [γ^{32}P]ATP without (1, 3) or with rottlerin (2, 4) as described in Experimental Procedures and separated by SDS−PAGE. The dried gel was exposed to Kodak film.

to PLD through different pathways, BK and S1P effects involved at least some common molecular mechanisms (e.g., Ca^{2+} influx). The present results confirm the role of Ca^{2+} in the regulation of PLD by BK, previously observed in airway smooth muscle (*20*) and are in accordance with the participation of Ca^{2+} in the signaling of S1P to PLD as we had defined in skeletal muscle cells (*29*). In contrast, stimulation of PLD by S1P in CFNPE9o$^-$ cells was independent of an increase in cytosolic Ca^{2+} (*10*).

PLD1 and PLD2 are widely distributed in mammalian tissues (*1*−*3*). Several studies show that PLD1 and PLD2 activities are modulated via common and specific regulators that can affect enzymatic activity directly or indirectly. Although studies performed with recombinant PLD2 appeared to show that the enzyme was constitutively active and regulated solely by inhibitory factors (*3, 39*), structure−function analysis of PLD2 revealed that it may be positively regulated by well-characterized activators of PLD1, such as ADP-ribosylation factors (*40, 41*) and PKCα (*42*). Many studies have shown that PLD1 is regulated in vitro and in vivo by the ~20-kDa GTP-binding protein RhoA (*1*−*3*, *42*); conversely, no evidence for PLD2 regulation by RhoA has

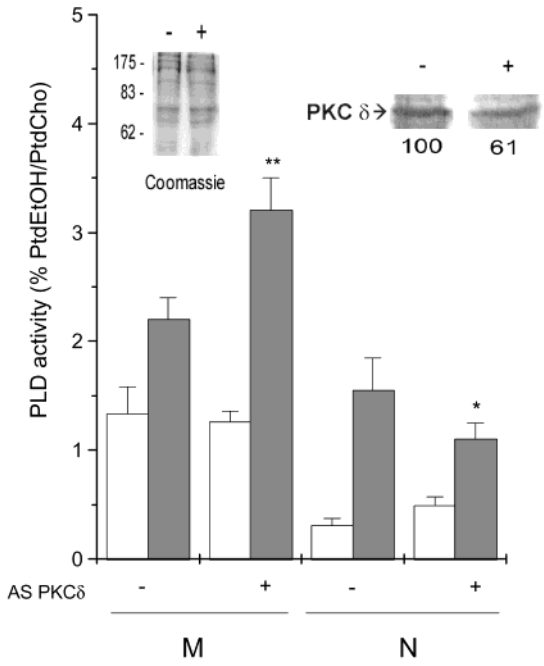

FIGURE 8: Effect of PKCδ-specific antisense oligodeoxynucleotides on BK activation of PLD in A549 cells. Total membrane (M) and nuclear (N) fractions prepared from A549 cells previously loaded with scrambled (−) or PKCδ-specific antisense (AS) ODN (+) and incubated without (empty bar) or with 1 μM BK (filled bar) were analyzed for PLD activity as described in the Experimental Procedures. Data are means ± SEM of values from four experiments. Effects of antisense ODN on BK stimulation were significant [(*) $p < 0.05$; (**) $p < 0.01$ by *t*-test]. (Inset) Coomassie staining and Western analysis of protein (20 μg) in total membrane fraction from A549 cells loaded with scrambled (−) or PKCδ-specific antisense (AS) ODN (+). Monoclonal anti-PKCδ antibodies were used for immunodetection. Amounts of PKCδ quantified by densitometry were recorded relative to control (scrambled ODN) = 100.

been provided. Moreover, in recent studies, PLD did not coimmunoprecipitate with RhoA and did not interact with it in two-hybrid assays or become activated by it, consistent with the view that PLD2 is not responsive to RhoA (*41*, *43*). Regarding the subcellular localization of PLD, in cells overexpressing the two isoforms, PLD1 was found in a perinuclear distribution (*4*, *44*), with PLD2 primarily in the plasma membrane (*4*) or adjacent vesicular compartments (*45*). Remarkably, we present evidence here that a significant percentage of total basal PLD activity in cells is in nuclei, and that PLD1 is the only isoform present in nuclei. In contrast, PLD2 was the predominant isoform found in the membrane fraction.

In our study, nuclear PLD activity was enhanced by BK and S1P, consistent with a report that nuclear PLD activity was stimulated by thrombin (*46*). Altogether, these findings support a role for PLD in nuclear envelope signal transduction. In light of the presence of PLD1, but not PLD2, in nuclei, A549 cells may be a useful system for further investigation of PLD function in nuclear lipid remodeling, which may be associated with cell division (*47*).

Previous studies in this laboratory described a mechanism of PLD activation in A549 cells by BK and S1P that involves RhoA, is insensitive to brefeldin A, and is likely localized in a specific subcellular compartment (*23*). Further insights into S1P- and BK-initiated signaling pathways are now provided by evaluation of agonist-induced PLD activation in subcellular fractions. Remarkably, the effectiveness of rottlerin to reduce nuclear PLD1 activation by BK and S1P strongly suggests the involvement in the process of PKCδ, a Ca^{2+}-independent PKC isoform, reported to play an important role in cell differentiation and growth inhibition (*48*). In this regard, it must be noted that other enzymes, such as PKCθ and calmodulin-dependent protein kinase III, are known to be efficaciously blocked by rottlerin (*49*), however, unlike PKCδ, they have never been implicated in PLD regulation. Regarding the molecular mechanisms involved in PKCδ activation by the two agonists, the increase in inositol phosphate level induced by BK and S1P was not paralleled by appreciable diacylglycerol accumulation (*50*), suggesting that the lipid plays a marginal role, if any, in PKCδ activation. It can be postulated that the already demonstrated dependence of PKCδ translocation/activation on tyrosine-phosphorylation events (*10*) takes place also in this cellular setting. In membrane fractions, PKCδ had different effects on BK and S1P signaling to PLD. Inhibition of PKCδ enhanced BK-stimulated PLD activity, while it reduced the S1P effect. Experimental evidence has already been provided for a positive role of PKCδ in S1P-stimulated PLD activity (*10*, *25*). However, our data obtained in preparations of total membranes with inhibition of PKCδ by rottlerin or antisense ODN are evidence of a role for PKCδ as negative regulator of PLD activation by BK in A549 cells. These observations are in agreement with a report that rottlerin enhanced basal PLD activity in rat fibroblasts (*13*). Information on the mechanisms involved in the PKCδ effects on PLD activity is controversial. Multiple in vitro experiments have shown that PLD can interact directly with various PKC isoforms (*1*−*3*), including PKCδ (*51*), and becomes activated in an ATP-independent manner through protein−protein interactions (*11*). However, given that the S1P effect, as well as BK action in nuclei, was reduced by rottlerin which inhibits kinase activity, phosphorylation-dependent events may be involved in agonist-induced activation of PLD in A549 cells. This hypothesis is supported by the recent finding in CFNPE9o$^-$ epithelial cells that PLD1 becomes phosphorylated via a PKCδ-dependent mechanism in response to S1P (*10*). The molecular basis for the inhibitory effect of PKCδ on BK stimulation of PLD in membranes is also unknown. It seems likely that PLD2 is the isoform whose activation is inhibited by PKCδ, since PKCδ had a stimulatory effect on enzymatic activity in nuclei, where only PLD1 is found. We reported, in agreement with Han et al. (*15*), that PLD2 was phosphorylated by PKCδ in vitro. Although PLD2 may be directly phosphorylated by PKCδ, PLD2 activity did not change after phosphorylation. Then, it remains to be determined whether phosphorylation-induced conformational changes affect PLD2 catalytic activity in A549 cells or whether the phosphorylated-PLD2 isoform interacts differently with its positive or negative regulator(s). Notably, recombinant PLD1 was not a substrate for PKCδ in in vitro experiments, strongly suggesting that the PKCδ-dependent activation of PLD reported here, as well as the similar findings obtained in CFNPE9o$^-$ cells (*10*), involve PKCδ only indirectly and rely on a putative protein kinase downstream of PKCδ.

In our study, BK and S1P differed also in their effects on distribution of RhoA. Both S1P and BK had similar effects on RhoA accumulation in nuclei, whereas S1P enhanced

RhoA translocation to membranes to a much greater extent than did BK. The observations that RhoA-sensitive PLD1 was the only isoform present in A549 cell nuclei and RhoA was translocated to this compartment in response to BK or S1P, are consistent with our previous finding on the role of RhoA in agonist-stimulation of PLD in these cells (*23*). They also implicate nuclei as the subcellular compartment in which this regulation occurs. A role for RhoA in the control of basal PLD activity in isolated nuclear fractions has been reported (*52*, *53*). In membranes, on the other hand, the coexistence of PLD1 and PLD2, the different effects of agonists on translocation of RhoA, and the opposing effects of PKC*δ* on stimulation of PLD activity by BK and S1P are all consistent with different PLD isoforms as targets of the two agonists. In particular, S1P signaling leading to PLD activation in membranes is compatible with the stimulation of PLD1, through RhoA- and PKC*δ*-dependent pathways, whereas the stimulatory effect of BK on PLD activity can best be explained by a different target, perhaps PLD2. Our observations in A549 airway epithelial cells extend the understanding of molecular targets of BK and S1P that participate via alternative GTP-binding protein- and PKC*δ*-dependent signaling pathways to regulation of PLD in specific subcellular fractions.

REFERENCES

1. Exton, J. H. (1999) *Biochim. Biophys. Acta 1439*, 121−133.
2. Liscovitch, M., Czarny, M., Fiucci, G., and Tang, X. (2000) *Biochem. J. 345*, 401−415.
3. Exton, J. H. (2002) *Rev. Physiol. Biochem. Pharmacol. 144*, 1−94.
4. Colley, W. C., Sung, T.-C., Roll, R., Jenco, J., Hammond, S. M., Altshuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) *Curr. Biol. 7*, 191−201.
5. Hammond, S. M., Altshuller, Y. M., Sung, T.-C., Rudge, A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) *J. Biol. Chem. 270*, 29640−29643.
6. Kiss, Z., Crilly, K. S., and Anderson, W. H. (1997) *Biochem. J. 328*, 383−391.
7. Lopez, I., Arnold, R. S., and Lambeth, J. D. (1998) *J. Biol. Chem. 273*, 12846−12852.
8. Slaaby, R., Du, G., Altshuller, Y. M., Frohman, M. A., and Seedorf, K. (2000) *Biochem. J. 351*, 613−619.
9. Kim, Y., Han, J. M., Han, B. R., Lee, K. A., Kim, J. H., Lee, B. D., Jang, I. H., Suh, P. G., and Ryu, S. H. (2000) *J. Biol. Chem. 275*, 13621−13627.
10. Ghelli, A., Porcelli, A. M., Facchini, A., Hrelia, S., Flamigni, F., and Rugolo, M. (2002) *Biochem. J. 366*, 187−193.
11. Kotter, K., Ji, A. S., von Eichel-Streiber, C., Park, J. B., Ryu, S. H., and Klein, J. (2000) *Biochim. Biophys. Acta 1485*, 153−162.
12. Melendez, A. J., Harnett, M. M., and Allen, J. M. (2001) *Curr. Biol. 11*, 869−874.
13. Hornia, A., Lu, Z., Sukezane, T., Zhong, M., Joseph, T., Frankel, P., and Foster, D. A. (1999) *Mol. Cell. Biol. 19*, 7672−7680.
14. Watanabe, H., and Kanaho, Y. (2000) *Biochim. Biophys. Acta 1495*, 121−124.
15. Han, J. M., Kim, J. H., Lee, B. D., Lee, S. D., Kim, Y., Jung, Y. W., Lee, S., Cho, W., Ohba, M., Kuroki, T., Suh, P. G., and Ryu, S. H. (2002) *J. Biol. Chem. 277*, 8290−8297.
16. Singer, W. D., Brown, A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem. 66*, 475−509.
17. Waite, K. A., Wallin, R., Qualliotine-Mann, D., and McPhail, L. (1997) *J. Biol. Chem. 272*, 15569−15578.
18. Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G. (2000) *J. Biol. Chem. 275*, 23911−23918.
19. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., Chen, J. (2001) *Science 294*, 1942−1945.
20. Pyne, S., and Pyne, N. J. (1995) *Biochem. J. 311*, 637−642.
21. Clark, K. J., and Murray, A. W. (1995) *J. Biol. Chem. 270*, 7097−7103.
22. Vasta, V., Meacci, E., Romiti, E., Farnararo, M., and Bruni, P. (1998) *Biochim. Biophys. Acta 1391*, 280−286.
23. Meacci, E., Vasta, V., Moorman, J. P., Bobak, D., Bruni P., Moss, J., and Vaughan, M. (1999) *J. Biol. Chem. 274*, 18605−18612.
24. Desai, N. N., Zhang, H., Olivera, A., Mattie, M. E., and Spiegel, S. (1992) *J. Biol. Chem. 267*, 23122−23128.
25. Meacci, E., Vasta, V., Donati, C., Farnararo, M., and Bruni, P. (1999) *FEBS Lett. 457*, 184−188.
26. Orlati, S., Porcelli, A. M., Hrelia, S., Van Brocklyn, J. R., Spiegel, S., and Rugolo, M. (2000) *Arch. Biochem. Biophys. 375*, 69−77.
27. Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J., Thangada, S., Liu, C. H., Hla, T., and Spiegel, S. (1998) *J. Cell Biol. 142*, 229−240.
28. Meacci, E., Donati, C., Cencetti, F., Oka, T., Komuro, I., Farnararo, M., and Bruni, P. (2001) *Cell. Signalling 13*, 593−598.
29. Meacci, E., Becciolini, L., Nuti, F., Donati, C., Cencetti, F., Farnararo, M., and Bruni, P. (2002) *FEBS Lett. 521*, 200−204.
30. Marcil, J., Harbour, D., Naccache, P. H., and Bourgoin, S. (1997) *J. Biol. Chem. 272*, 20060−20064.
31. Houle, M. G., and Bourgoin, S. (1999) *Biochim. Biophys. Acta 1439*, 135−150.
32. Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., and Shields, D. (2001) *Mol. Biol. Cell 12*, 943−955.
33. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) *Nature 348*, 334−336.
34. Little, L., Alcouloumre, B., Drotar, A. M., Herman, S., Robertson, R., Yeh, R. Y., and Miller, A. L. (1987) *Biochem. J. 248*, 151−159.
35. Bligh, E. A., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
36. Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1592−1596.
37. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell 75*, 1137−1144.
38. Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kuzumaki, N., and Nozawa, Y. (1999) *J. Biol. Chem. 274*, 27385−27391.
39. Lee, C., Kim, S. R., Chung, J.-K., Frohman, M. A., Kilimann, M. W., and Rhee, S. G. (2000) *J. Biol. Chem. 275*, 18751−18758.
40. Lopez, I., Arnold, R. S., and Lambeth, J. D. (1998) *J. Biol. Chem. 273*, 12846−12852.
41. Sung, T.-C., Altshuller, Y. M., Morris, A. J., and Frohman, M. A. (1999) *J. Biol. Chem. 274*, 494−502.
42. Slaaby, R., Du, G., Altshuller, Y. M., Frohman, M. A., and Seedorf, K. (2000) *Biochem. J. 351*, 613−619.
43. Du, G., Altshuller Y. M., Kim, Y., Han, Y. M., Ryu, S. H., Morris, A. J., and Frohman, M. A. (2000) *Mol. Biol. Cell 11*, 4359−4368.
44. Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R., and Wakelam, M. J. (1998) *Curr. Biol. 8*, 835−838.
45. Divecha, N., Roefs, M., Halstead, J. R., D'Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K. M., Wakelam, M. J. O., and D'Santos, C. (2000) *EMBO J. 19*, 5440−5449.
46. Baldassarre, J. J., Jarpes, M. B., Alferes, L., and Raben, D. M. (1997) *J. Biol. Chem. 272*, 4911−4914.
47. Rose, K, Rudge, S. A., Frohman, M. A., Morris, A. J., and Engebrecht, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12151−12155.
48. Gschwendt, M. (1999) *Eur. J. Biochem. 259*, 555−564.
49. Gschwendt, M., Muller, H. J., Kielbassa, K., Zang, R., Kittstein, W., Rinck, G., and Marks, F. (1994) *Biochem. Biophys. Res. Commun. 199*, 93−98.
50. Vasta, V., Meacci, E., Catarzi, S., Farnararo, M., and Bruni, P. (1998) *Biochem. Soc. Trans. 26*, S229.
51. Hodgkin, M. N., Clark, J. M., Rose, S., Saqib, K., and Wakelam, M. J. O. (1999) *Biochem. J. 339*, 87−93.
52. Balboa, M. A., and Insel, P. A. (1995) *J. Biol. Chem. 270*, 29843−29847.
53. Banno, Y., Tamiya-Koizumi, K., Oshima, H., Morikawa, A., Yoshida, S., and Nozawa, Y. (1997) *J. Biol. Chem. 272*, 5208−5213.

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