In HL60 cells, the membrane-bound phospholipase D (PLD) was stimulated by 4\(\alpha\)-phorbol 12-myristate 13-acetate (PMA) in the presence of the cytosolic fraction from HL60 cells or rat brain. The cytosolic factor for this PMA-induced PLD activation was subjected to purification from rat brain by sequential chromatographies. The PLD stimulating activity was found in protein kinase C (PKC) fraction containing \(\alpha\), \(\beta\), \(\beta'I\), and \(\gamma\) isozymes. PKC \(\alpha\) and -\(\beta\), but not \(\gamma\), isozymes were found to activate membrane-bound PLD. PKC \(\alpha\) was much more effective than PKC \(\beta\) for PLD activation. Millimolar concentrations of MgATP were required for the PKC-mediated PLD activation in HL60 membranes. MgATP is utilized to maintain the levels of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) under these assay conditions. The PKC-mediated PLD activation was completely inhibited by neomycin, a high affinity ligand for PIP\(_2\), and this suppression was recovered by the addition of exogenous PIP\(_2\). Thus, these results suggest that PIP\(_2\) is supposed to play a key role in PKC-mediated PLD activity in HL60 membranes. Furthermore, PKC\(\alpha\)-mediated PLD activation was potentiated by the addition of recombinant RhoA protein in the presence of guanosine 5'-O-(3-thiotriphosphate) (GTP\(_\beta\)S). The results obtained here indicate that PKC\(\alpha\) and RhoA (GTP form) exert a synergistic action in the membrane-bound PLD activation in HL60 cells.

Phospholipase D (PLD)\(^1\) has been recognized to play an important role in signal transduction of many types of cells. PLD hydrolyzes phosphatidylcholine (PC) to generate phosphatic acid (PA) and choline (1). PA and its dephosphorylated product diacylglycerol are important second messengers. PLD is activated in a variety of cells in response to receptor agonists, and the implication of two small GTP-binding proteins, ADP-ribose-pyrophosphatase I (ARF) and Rho, in the regulation of PLD is presented by the observations that PKC up-regulates PLD activity (4, 5). This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The present study was designed to gain more insight into the mechanisms underlying the PMA-induced PLD activation in HL60 membranes. A cytosolic factor reconstituting PMA-induced PLD activity was resolved as PKC fraction from rat brain. Among PKC\(\alpha\), \(\gamma\), and \(\gamma\) isozymes, PKC\(\alpha\) was the most effective in activating membrane-bound PLD. PKC\(\alpha\)-mediated PLD activation was synergistically stimulated by RhoA in the presence of GTP\(_\beta\)S. Furthermore, MgATP and phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) were necessary for the membrane-bound PLD activation by the partially purified PKC

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**SYNERGISTIC ACTION OF SMALL GTP-BINDING PROTEIN RhoA*\(^\dagger\)**

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\(^1\)The abbreviations used are: PLD, phospholipase D; PBut, phosphatidylbutanol; PKC, protein kinase C; PMA, 4\(\alpha\)-phorbol 12-myristate 13-acetate; 4\(\alpha\)-PDD, 4\(\alpha\)-phorbol 12,13-didecanoate; GTP\(_\gamma\)S, guanosine 5'-O-(3-thiotriphosphate); GDP\(_\beta\)S, guanosine 5'-O(2-thiotriphosphate); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatic acid; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride; ARF, ADP-riboseylation factor; GDI, GDP dissociation inhibitor; GST, glutathione S-transferase.

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fraction which was free from small GTP-binding proteins of brain cytosol.

**EXPERIMENTAL PROCEDURES**

Materials—HL60 human promyelocytic leukemia cell line was kindly supplied by Dr. T. Okazaki (Osaka Dental University). [3H]Oleic acid and [3H]PDDP were obtained from DuPont NEN, myo-[3H]inositol and [γ-32P]JATP were from Amersham. PMA, 4a-PDD and PIP2 were from Sigma. GTP-s was from Boehringer Mannheim. Antibodies against PKC isozymes (α, β, γ, δ, ε, θ, and z) and small GTP-binding proteins (RhoA, Rac1/C42Hs) and RhoGDI were from Santa Cruz Biotechnology. Silica Gel 60 (LK6D) plates were from Whatman. Mono Q and Superose 12 columns were from Pharmacia Biotech Inc. Hydroxylapatite was from Mitsui-Toshu Chemicals. Protein determination reagents were from Bio-Rad. Recombinant RhoA and GST-RhoGDI were kindly supplied by Dr. Y. Takai (Osaka University).

Cell Culture and Cell Labeling—HL60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were grown at a cell density of 0.2–1.0 × 10⁶ cells/ml in a humidified atmosphere containing 5% CO₂ at 37°C. For assay of PLD activity, cells were labeled with [3H]Oleic acid (0.5 μCi/ml) for 12–15 h. Under these conditions, 60–65% of total radioactivity incorporated into cells was found in the phosphatidylserine (PS) fraction. For the analysis of phosphoinositides, cells were labeled with myo-[3H]inositol (2 μCi/ml) in inositol-free medium supplemented with 2 μM glutamine and 3.5 mg/ml bovine serum albumin for 24 h.

Preparation of Membranes and Cytosol Fractions from HL60 Cells—Membranes and cytosol fractions were prepared by the method described above (3), with minor modifications. The labeled cells were washed twice with buffer A (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 0.5 mM MgATP, 1 mM EGTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin) and resuspended in buffer A. Cells were then disrupted by N₂ cavitation (600 p.s.i. at 4°C for 30 min). After broken cells were removed by centrifugation at 900 × g for 5 min, membrane and cytosol fractions were separated by centrifugation at 100,000 × g for 60 min. Membranes were washed once and resuspended in buffer A. Membrane fractions were used within 12 h after isolation. Cytosol proteins were concentrated using Centricon 10 (Amicon) and stored at −80°C until use.

Assay of PLD Activity in HL60 Membranes—[3H]Oleic acid-labeled HL60 membranes (50 μg of protein/assay) and crude cytosol or purified PKC fractions were incubated in buffer A containing CaCl₂ to give a final free Ca²⁺ concentration of 1 μM (total 0.1 ml) and were stimulated with 100 nM PMA and/or 10 μM GTP-s at 37°C for 15 min in the presence of butanol (0.3%, v/v). Reactions were terminated by the addition of chlorform/methanol (1:2, v/v). Lipids were extracted according to Bligh and Dyer (21) and separated on Silica Gel 60 TLC plates, impregnated with chloroform/methanol/concentrated HCl (20:40:1, v/v). Lipids were extracted and separated on Silica Gel 60 TLC plates in a solvent system of the upper phase of ethyl acetate/2,4-trimethylpentane/acetic acid/water (13:2:3:10, v/v) as described previously (22). The plates were exposed to iodine vapor, and [3H]JPBut was identified by comigration with PBut standard which was prepared by the partially purified cabbage PLD (23). The spots scraped off the plates were mixed with scintillation mixture, and the radioactivity was counted in a liquid scintillation counter (Beckman LS-6500). To measure PLD activity using exogenous substrate, assays were carried out with phosphatidylethanolamine (PE)/PIP2/[3H]DDPC essentially according to the method of Brown et al. (9).

Analysis of Phosphoinositides in HL60 Membranes—myo-[3H]inositol-labeled HL60 membranes (50 μg of protein/assay) were prepared as described above and were incubated under the same conditions of PLD assay except butanol. Reactions were terminated by the addition of chlorform/methanol/concentrated HCl (20:40:1, v/v). Lipids were extracted and separated on Silica Gel 60 TLC plates, impregnated with 1.2% potassium oxalate in a solvent system of chlorform/methanol/28% ammonia water/water (45:40:5:8, v/v) as described previously (24). The spots corresponding to inositol phosphates ([3H]IP₃) were visualized by autoradiography with PIP₃ standard. The radioactivity of spots was counted as described above. In another set of experiments, HL60 membranes (50 μg of protein) were incubated with [γ-32P]JATP (10 μCi/ml) in the presence or absence of 0.5 mM ATP at 37°C for 15 min. [3H]JPBut was analyzed as described above or by autoradiography.

Separation of PKC Isozymes and Small GTP-binding Proteins from Rat Brain Cytosol—Rat brains (approximately 70 g) were homogenized in buffer B (25 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EGTA, 1 mM PMSF, 50 mM 2-mercaptoethanol, and 10 μg/ml leupeptin) with Polytron homogenizer (Kinematica). The homogenate was centrifuged at 100,000 × g for 60 min to obtain cytosolic fraction. The supernatant was loaded onto a Mono Q column equilibrated with buffer C (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol). Proteins were eluted with linear gradient of NaCl (0–0.7 M) in buffer C using first protein liquid chromatography (Pharmacia). The PKC activity peak eluted with 0.2–0.3 M NaCl was then applied to a Superose 12 column equilibrated with buffer C containing 150 mM NaCl and eluted with the same buffer. The PKC activity peak was pooled and then was applied to a hydroxypatite column equilibrated with buffer D (20 mM KPO₄, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol). Proteins were eluted with linear gradient of potassium phosphate (20–300 mM in buffer D). Individual fractions from each chromatography were subjected to Western blot analysis using antibodies against PKCa, -β, -γ, PLD, and γ isozymes and small GTP-binding proteins.

Assay of Protein Kinase C Activity—The PKC activity was assayed as described previously (25) by using myelin basic protein (MBP) as a substrate (26). The reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM CaCl₂, 10 mM PMA, 50 μg/ml phosphatidyserine, 10 μM [γ-32P]JATP, and 50 μg/ml MBP. After incubation at 30°C for 5 min, reactions were terminated with 50 μl of 20 mM ATP. A 50-μl aliquot was spotted on P81 phosphocellulose paper, and then the paper was washed with 75 mM phosphoric acid. The radioactivity retained on the paper was determined by Cerenkov counting. One unit of PKC was expressed as 1 pmol of [γ-32P]JATP incorporated into MBP/5 min.

Translocation of PKCs and Small GTP-binding Proteins to Membrane—After incubation under the same conditions as the PLD assay, the reaction mixture was centrifuged at 100,000 × g for 30 min to obtain the membrane pellet. Membranes were washed once in buffer E (20 mM Tris-HCl, pH 7.4, 30 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF, and 10 μg/ml leupeptin) and resuspended in buffer E containing 1% Triton X-100. After incubation at 4°C for 60 min, the suspension was centrifuged at 100,000 × g for 60 min to obtain the membrane extract. Aliquots mixed with Laemmli’s sample buffer (27) were subjected to electrophoresis and Western blot analysis.

Electrophoresis and Western Blot Analysis—Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8 or 12% polyacrylamide gels for PKC or small GTP-binding protein, respectively, according to the method by Laemmli (27). Proteins were electrophoretically transferred onto nitrocellulose membrane (28). Blocking was performed in Tris-buffered saline containing 5% skimmed milk powder and 0.05% Tween 20. Western blot analysis using specific antibodies was performed as described previously (29).

**RESULTS**

Requirement of Cytosolic Components for Activation of Membrane-bound PLD in HL60 Cells—Recent reconstitution studies in the cell-free system and cytosol-depleted permeabilized cells have shown that several cytosolic factors are required for activation of PLD by GTPγS and/or PMA (2). We examined the effects of cytosolic fractions separated from HL60 cells or rat brain on the membrane-bound PLD activity from HL60 cells in response to PMA and/or GTPγS (Fig. 1). Weak PLD activity was detected in both cytosolic fractions, when measured using the PE/P3/[3H]DDPC (16:1:4:1) substrate system as described by Brown et al. (9). However, the cytosolic PLD activity was stimulated at most 2-fold with both 100 nM PMA and 10 μM GTPγS (data not shown). In the absence of cytosolic fraction, little membrane-bound PLD activity was detected in response to either PMA or GTPγS. However, when the cytosolic fractions from HL60 cells or rat brain were included in the reaction mixture, nearly 5-fold enhancement of PLD activity was seen in response to either 100 nM PMA or 10 μM GTPγS. Furthermore, in the presence of both PMA and GTPγS, the enhancement (more than 15-fold) of membrane-bound PLD activity was much greater than that observed in response to either stimulant alone.

The formation of phosphatidylalcohol such as PBut is commonly utilized to monitor PLD activity (1, 2). Although [3H]PBut has been reported to be metabolically stable, its degradation in various assay conditions is not clearly known. We
have then analyzed the degradation of [3H]PBut during the course of experiments. [3H]PBut was prepared from PMA-stimulated intact HL60 cells. The level of [3H]PBut decreased only very little (less than 10% statistically not significant) in the reaction buffer during incubation (15 min). Additionally, membrane fraction, cytosolic fraction, and stimulators (PMA and GTPyS) were almost without effect (statistically not significant) on PBut degradation in our incubation condition. Therefore, we examined PLD activity by the formation of [3H]PBut.

Resolution of PMA-dependent PLD Activating Fractions from Rat Brain Cytosol—The PMA- or GTPyS-induced PLD activation in HL60 membranes was dependent on rat brain cytosol (Fig. 2A). To separate the PMA- and GTPyS-dependent cytosolic factors responsible for membrane-bound PLD activation, the brain cytosol was subjected to Mono Q anion exchange chromatography (Fig. 3A). The fractions enhancing PMA- and/or GTPyS-induced PLD activity in HL60 membranes showed a broad peak. Unadsorbed fractions were ineffective in stimulating PLD activity. The active peak enhancing the PMA-induced PLD activity (fractions 35–55) was found to overlap with the peak of PKC activity (Fig. 3A). However, column fractions 32–41 also could augment the GTPyS-induced PLD activity (Fig. 3A). A small GTP-binding protein RhoA was detected in these fractions by Western blot analysis (Fig. 3A, inset). The fractions 42–55 (PKC-rich fractions) were pooled and concentrated. However, this PKC-rich fraction (Mono Q-PKC) still had an ability to increase GTPyS-induced PLD activity (Fig. 2B), suggesting the coexistence of small GTP-binding proteins. In order to separate PKCs from small GTP-binding proteins, the Mono Q-PKC fraction was subjected to Superose 12 gel filtration chromatography. The PKC fraction eluted at a position of about 80 kDa was separated from the fraction containing small GTP-binding proteins (less than 80 kDa) and concentrated. This PKC fraction (Superose-PKC) contained α, β, and γ isozymes, but Rho family small GTP-binding proteins, RhoA, Rac1/Rac2, and Cdc42Hs were undetectable by Western blot analysis (data not shown). The Superose-PKC fraction stimulated PLD activity in response to PMA but failed to fulfill the stimulatory effect of GTPyS (Fig. 2C). PLD activity was not detectable in this PKC fraction using the PE/PIP2/[3H]DPPC (16:1:4:1) substrate system (data not shown) as described by Brown et al. (9), although crude rat brain cytosol contained weak PLD activity as described above.

Activation of Membrane-bound PLD of HL60 Cells by Partially Purified PKC from Rat Brain—To examine the mechanism of PKC-mediated PLD activation, isolated HL60 membranes and the Superose-PKC fraction obtained after gel filtration were used. In the presence of the Superose-PKC fraction, PLD activity by PMA was concentration-dependent and reached the maximal level at around 100 nM PMA (Fig. 4A). An inactive phorbol ester, 4a-PDD failed to activate PLD (Fig. 4A). The PKC-mediated PLD activation was time-dependent and reached a plateau at 10–15 min after addition...
of the PKC fraction (Fig. 4B). Incubation of the HL60 membranes with the PKC fraction in the presence of 100 nM PMA caused translocation of PKCα, β1, and βII isozymes to membranes as inferred by Western blot analysis (Fig. 4C). The time course of PKC translocation (Fig. 4D) showed a good correlation with PMA-induced PLD activation (Fig. 4B).

Effect of MgATP and Phosphatidylinositol 4,5-Bisphosphate on PKC-mediated PLD Activity—The enhancement by MgATP of PLD activation in response to GTPγS was demonstrated in many types of cells (15, 30–34). The effect of MgATP on PKC-mediated PLD activity was examined in HL60 membranes. Although no stimulatory effect on the PKC-mediated PLD activity was observed at concentrations less than 50 μM, MgATP greatly potentiated PKC-mediated PLD activity, and the maximal effect was obtained at 0.5 mM (Fig. 5A). Recent reports (9, 35) have indicated evidence that PIP2 functions as a cofactor for PLD activity. We examined the effect of MgATP on the level of [3H]PIP2 in membranes prepared from myo-[3H]inositol-labeled HL60 cells. As shown in Fig. 5B, the level of [3H]PIP2 decreased in the absence of MgATP. In contrast, 0.5 mM MgATP caused an increase in the level of [3H]PIP2. PMA alone or both PMA and partially purified PKC fraction had no stimulatory effects on the level of [3H]PIP2. Additionally, the incubation of HL60 membranes with [γ-32P]ATP in the presence of 0.5 mM MgATP led to incorporation of 32P into PIP2 (data not shown). Similar observations have been observed in rat brain membranes (36) and permeabilized U937 cells (37). These results suggest that the effect of MgATP is likely due to its ability to maintain the level of PIP2.

In order to further assess the involvement of PIP2 in PMA-mediated PLD activation, we examined the effect of neomycin, which binds polyphosphoinositides with high affinity. However, neomycin is reported to inhibit PKC activity at high concentrations (more than 2 mM) (38). Therefore, the HL60 membrane fraction was treated with neomycin (less than 1 mM) and then excess neomycin was washed out prior to stimulation of partially purified PKC fraction and PMA. The PKC-mediated PLD activity in HL60 membranes was suppressed in parallel to increasing concentrations of neomycin (Fig. 6). 1 mM neomycin caused a complete inhibition (approximately 95%) of the PLD activity. However, the suppressed PKC-mediated PLD activity by 1 mM neomycin was restored by addition of PIP2 in a concentration-dependent manner (Fig. 6). In these experiments, PIP2 was mixed with phosphatidylethanolamine (PIP2/PE, 1:5 mol/mol), because of effective incorporation into membranes. PE alone had no effect on restoring PLD activity in neomycin-treated membranes (data not shown).

PKC Isozymes Responsible for Activation of Membrane-bound PLD—The Superose-PKC fraction from rat brain cytosol capable of activating PLD contained α, β, βII, and γ isoforms. In order to determine which isoform was involved in PLD activation, the PKC fraction was further subjected to hydroxylapatite column chromatography; the PKC fraction after Su-
perose 12 gel filtration was applied to a hydroxylapatite column and eluted by a linear concentration gradient of potassium phosphate. As shown in Fig. 7A, two major fractions capable of enhancing PMA-induced PLD activity were separated. Western blot analysis revealed that the first peak (peak 1) contained PKCα (α plus β1) and PKCe was present in the second peak (peak 2). PKCy was found in fractions 5–15 (Fig. 7B). Each PKC isozyme fraction: PKCy (fractions 5–15), PKCβ (fractions 20–35), and PKCa (fractions 50–70) was separately pooled and concentrated.

The effects of these purified PKC isozymes on HL60 membrane PLD activity were examined. In the presence of PMA (100 nM), PLD activity was enhanced by PKCe or -β in a concentration-dependent manner (Fig. 8A and B). PKCa was the most effective with a maximal effect obtained at about 10 units/assay. The maximal PLD activity obtained at around 10 units/assay of PKCβ was almost half that induced by PKCe. PKCy had no effect on PLD activation in HL60 membranes (data not shown). The PLD activity stimulated by PKCa (2.5 units/assay) plus β (2.5 units/assay) was the same as that obtained with PKCa alone (Fig. 8C). GTPγS in the absence of PMA did not stimulate PLD activity. However, in the presence of both PMA and GTPγS, the PKC-mediated PLD activity was synergistically potentiated (Fig. 8). Additionally, recombinant PKCe stimulated the membrane-bound PLD activity in HL60 cells in a quite similar manner as observed with the purified PKCα fraction (data not shown).

Effect of Small GTP-binding Protein RhoA on PKCα-mediated PLD Activation—Recently, we have demonstrated possible evidence that PKC-mediated PLD activity in HL60 membranes could be enhanced by RhoA in the presence of GTPγS (20). The PLD activity of HL60 membranes was stimulated by recombinant GTPγS-bound RhoA alone in a concentration-dependent manner (Fig. 9A). RhoA at 20 nM caused a remarkable activation of the PKCα-mediated PLD of HL60 membranes in the presence of both PMA and GTPγS (Fig. 9B). Incubation of HL60 membranes with HL60 cytosol in the presence of PMA or GTPγS caused translocation of PKCa and RhoA to membranes, respectively, as inferred by Western blot analysis (Fig. 10). Cdc42Hs also was translocated to membranes although the extent was much less than that of RhoA (data not shown). Another Rho family member, Rac, was undetectable in either cytosol or membrane of HL60 cells as mentioned previously by Siddiqi et al. (39).

**DISCUSSION**

Several factors have been implicated in the regulation of PLD activity, such as Ca²⁺, PKC, protein-tyrosine kinase, and GTP-binding proteins (2). However, their detailed mechanisms are not fully understood. Recently, PLD assay systems using permeabilized cells or cell-free preparations have been developed, and cytosolic factors including ARF and Rho protein are identified as regulatory factors for PLD activity. PMA, known as a PKC activator, activates PLD in many types of cells. Although the effect of PMA is assumed to be mediated through
the presence of Ca2+ as PKCs. Our previous report (20) has shown that the partially purified by sequential chromatographies and identified in rat brain. The cytosolic factors required for this activity were PMA in the presence of the cytosolic fractions from HL60 cells, the membrane-bound PLD activity was stimulated by PMA and GTPγS, or both PMA and GTPγS at 37°C for 15 min in the presence of PKCα (5 units) and/or RhoA (20 nM). PLD activity was determined to measure the formation of [3H]PBut as described under “Experimental Procedures.” Data represent the mean ± S.D. of two different experiments, each carried out in duplicate.

PKC, the pathway leading to PLD activation remains to be disclosed. In the present study, the mechanism of PMA-induced PLD activation was investigated in HL60 membranes. In the cell-free system using isolated membranes from HL60 cells, the membrane-bound PLD activity was stimulated by PMA in the presence of the cytosolic fractions from HL60 cells or rat brain. The cytosolic factors required for this activity were partially purified by sequential chromatographies and identified as PKCs. Our previous report (20) has shown that the PKC-mediated PLD activation was most effectively induced in the presence of Ca2+. In addition, PKCα, -β1, -βII, and lesser amounts of -ζ isozyme were observed, but PKCγ, -δ, -ε, -η, and -θ isozymes were undeetactable in HL60 cells by Western blot analysis (data not shown). These results suggest that conventional PKC (cPKC) isozymes may play a role in PMA-stimulated PLD activation in HL60 membranes. We have further examined PKC isozymes involved in the regulation of membrane-bound PLD. The results indicate that PKCα and -β activate the membrane-bound PLD and also that PKCα is much more effective in its activation in HL60 cells. The study of the regulation of PLD in membranes isolated from Chinese hamster lung (CCL39) fibroblasts demonstrated that addition of purified PKCα and -β from rat brain could activate PLD (8). In our study, PKCα and -β did not additively activate the PLD in HL60 membranes (Fig. 8C), suggesting that both PKC isozymes act at the same step for the PLD activation.

Previously, Tettenborn and Mueller (40) demonstrated that the PLD activation by PMA was dependent on the presence of ATP in HL60 cell lysates. Olson et al. (3) also reported that PMA-induced PLD activation was dependent on ATP in the neutrophil cell-free system. The PKC-mediated PLD activation in HL60 membranes required MgATP at millimolar concentrations (Fig. 5). Our previous study (20) has shown that the PKC-mediated PLD activation in HL60 membranes was not suppressed by Ro31-8425, a potent PKC inhibitor. Similar findings were reported by Conricode et al. (41) showing that membrane-bound PLD in CCL39 fibroblasts could be activated by PKC in a phosphorylation-independent mechanism and that the PLD activation by PKC was observed even in the absence of ATP, suggesting that PKC may activate PLD by an allosteric mechanism without ATP-dependent phosphorylation. On the other hand, it was reported more recently that the effect of ATP on PKC-mediated PLD activation is mediated by phosphorylation in human neutrophils (42). Although this discrepancy could reflect difference in cell type, our present data cannot exclude this possibility.

Several recent studies have provided evidence that PIP2 may act as an important cofactor for PLD activity. Brown et al. (9) developed a reconstitution system for solubilized PLD activity from HL60 cells in which the substrate PC was present in the form of mixed phospholipid micelles including PIP2 and demonstrated the requirement of PIP2 in the ARF-mediated PLD activation. Lisovitch et al. (35) have shown that the activity of partially purified PLD from brain membranes was stimulated considerably by PIP2. In permeabilized U937 cells (37), GTPγS was observed to elevate the levels of polyphosphoinositides in the presence of MgATP, and either GTPγS- or PMA-induced PLD activation was prevented by the antibody against phosphatidylinositol 4-kinase. Furthermore, neomycin, a high affinity ligand for PIP2, inhibited the activity of purified PLD from brain membranes (35) and GTPγS-induced PLD activation in permeabilized HL60 cells (15), human neutrophils (17), and U937 cells (37). The results obtained in the present study also indicated that PIP2 was required for PKC-mediated PLD activation in HL60 membranes. In fact, the level of PIP2 in HL60 membranes was increased by incubation with MgATP (Fig. 5B). Therefore, MgATP is supposed to play a key role in maintaining the level of PIP2 for PLD activity. The effect of PIP2 might be partly explained by the fact that PIP2 (100 μM) caused little enhancement (approximately 1.4-fold) of PKC activity (data not shown). However, at present, detailed information for the site of action of PIP2 and involvement of protein phosphorylation is not available and should be obtained by additional experiments which are currently in progress in our laboratory.

The PKC-mediated PLD activation in HL60 membranes was potentiated by the addition of GTPγS (Fig. 8). This finding led us to assume that translocated PKCα or -β interacts with membrane-associated GTP-binding proteins, resulting in a synergistic activation of PLD. Several lines of evidence are present to indicate that activation of PLD is mediated by small GTP-binding proteins which interact directly with the solubilized PLD (9, 39, 43–45). In addition, it was demonstrated that PIP2 synthesis is regulated by Rho family small GTP-binding proteins (46, 47). Our previous study (20) has shown that RhoGDI, Rho GDP dissociation inhibitor which extracts Rho proteins from membranes, prevented the synergistic effect by GTPγS in PKC-mediated PLD activation in HL60 membranes, and also that this suppressed PLD activation was restored by the addition of recombinant RhoA. These results suggest the
interaction between PKC and membrane-associated RhoA. Western blot analysis with the antibody to RhoA showed the presence of RhoA in the HL60 membrane fraction (data not shown). Furthermore, the present study showed that PKC-mediated PLD activity was potentiated by the addition of recombinant RhoA in the presence of GTP-γS (Fig. 9). It was also shown that cytosolic PKCα and RhoA were translocated to membranes in a PMA- and GTP-γS-dependent manner, respectively. Most recently, Siddiqi et al. (39) indicated that not only RhoA but also Rac and Cdc42Hs induced PLD activation in HL60 membranes. Among the Rho family GTP-binding proteins, however, only RhoA was translocated to HL60 membranes in the presence of GTP-γS (39). These findings suggest the involvement of RhoA in the regulation of membrane-bound PLD in HL60 cells.

Despite many studies, the exact mechanism for the implication of PKC in PLD activation has not yet been defined. It may be possible that PKC interacts directly with PLD in membranes or that PKC interacts with other membrane-associated proteins that in turn activates PLD. In the present study, the PKCα fraction free from small GTP-binding proteins was observed to activate the membrane-bound PLD (Fig. 8). However, GTP-γS synergistically enhanced PKC-mediated PLD activation. This synergistic activation by GTP-γS was prevented by pretreatment of HL60 membranes with RhoGD1, as shown in the previous study (20). Recent reports demonstrate that ARF-mediated PLD activation is modulated by other as yet unidentified cytosolic proteins (48–50). RhoA may act as a PKC-modulating factor in PKC-mediated PLD activation. We have recently obtained a preliminary finding that in the membranes pretreated with GDP/βS, the PKCα-mediated PLD activation by PMA was almost completely abolished. Thus, the PKC-mediated PLD activation mechanism involving RhoA appears to be more complex than expected. The reconstitution system using purified membrane-bound PLD will add further insight into the mode of the synergistic activation of membrane-bound PLD in HL60 cells.

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