Biochemical and Biophysical Research Communications **267,** 305–310 (2000)
doi:10.1006/bbrc.1999.1941, available online at http://www.idealibrary.com on IDEAL®

Downregulation of Phospholipase D by Protein Kinase A in a Cell-Free System of Human Neutrophils

Jong-Young Kwak*,[1] and David J. Uhlinger†
Department of Biochemistry, Dong-A University College of Medicine, Pusan 602-103, Korea; and †Department of Drug Discovery, R. W. Johnson Pharmaceutical Research Institute, Raritan, New Jersey 08869

Received November 17, 1999

Agents which elevate cellular cAMP are known to inhibit the activation of phospholipase D (PLD) in human neutrophils. The PLD activity of human neutrophils requires protein factors in both membrane and cytosolic fractions. We have studied the regulation of PLD by the catalytic subunit of protein kinase A (cPKA) in a cell-free system. cPKA significantly inhibited GTPγS-stimulated PLD activity but had no effect on phorbol ester-activated PLD activity. Pretreatment of plasma membranes with cPKA and ATP inhibited subsequent PLD activation upon reconstitution with untreated cytosol. RhoA, which is known to be a plasma membrane activator of PLD, was dissociated from PKA-treated plasma membrane by addition of cytosol. Plasma membrane-associated RhoA in human neutrophils was phosphorylated by cPKA. The PKA-phosphorylated form of RhoA was more easily extracted from membranes by RhoGDI than the unphosphorylated form. These results suggest that inhibition of neutrophil PLD by PKA may be due to phosphorylation of RhoA on the plasma membrane.

Phospholipase D (PLD) which is activated via fMet-Leu-Phe (fMLP) and by phorbol esters in neutrophils catalyzes the hydrolysis of phosphatidylcholine to generate free choline and phosphatidic acid (1, 2). Phosphatidic acid can be further metabolized by phosphatidic acid phosphohydrolase to form diacylglycerol and by phospholipase A_2 to form lysophosphatidic acid. The mechanism for activation of the respiratory burst and other functions in neutrophils may be linked to amplified generation of diacylglycerol through phospholipase C and PLD (3–5).

Many factors including Ca^{2+}, protein kinase C (PKC), G proteins and receptor or non-receptor tyrosine kinases have been implicated in the regulation of PLD (5–8). Cell-free systems have advanced our understanding of the regulation of PLD. A cell-free system of neutrophil PLD activation by GTPγS or phorbol 12-myristate 13-acetate (PMA) requires the participation of protein components both in the plasma membrane and in the cytosol (9). ADP ribosylation factor (ARF) was identified as a cytosolic factor that activates the PLD in HL-60 membranes (10, 11). In the neutrophil system, ARF acts synergistically with an essential 50-kDa cytosolic factor (12). One of the plasma membrane components is a Rho family small molecular weight G protein (13, 14). We have previously shown that RhoA is required to reconstitute PLD activity by neutrophil membrane (15). Neutrophil PLD is also activated by calcium-dependent forms of PKC and requires ATP, indicating that its activation by PKC is conventional and phosphorylation-dependent (16).

cAMP interferes with fMLP activation of neutrophil responses including O_2^- generation and degranulation. PLD activation by fMLP is blocked with cAMP-increasing agents such as dibutyryl cAMP, prostaglandin E, and theophylline but the activation by phorbol esters is not affected by these agents (17, 18). fMLP binding to neutrophils was not inhibited by pretreatment of the cells with the cAMP-increasing agents, suggesting that the target of inhibition is downstream of the receptor (19, 20). It has been also suggested that the target of phosphorylation may be a site proximal to PLD because PLD stimulated by different agonists showed differential responses to cAMP-increasing agents (17, 18). The mechanism of inhibition of neutrophil PLD by cAMP is not well understood.

Abbreviations used: ARF, ADP ribosylation factor; cPKA, catalytic subunit of protein kinase A; fMLP, formyl-methionyl-leucyl-phenylalanine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); PEth, phosphatidylethanol; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; RhoGDI, Rho GDP dissociation inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[1] To whom correspondence should be addressed at Department of Biochemistry, Dong-A University College of Medicine, Seo-Ku, Pusan 602-103, South Korea. Fax: 82-51-241-6940. E-mail: jykwak@seunghak.donga.ac.kr.

0006-291X/00 $35.00

cAMP acts through cAMP-dependent protein kinase (PKA). The mechanisms by which cAMP inhibits enzyme activity in intact cells can be studied using the catalytic subunit of PKA (cPKA) in a cell-free system. To decipher the function of PKA in downregulation of PLD activation it will be essential to identify the target of PKA. The purpose of the present paper is to utilize a cell-free system to identify signaling pathways responsible for the downregulation of PLD by PKA. We found that the activation of PLD is inhibited by PKA in a cell-free system and this downregulation may be due to phosphorylation of RhoA by PKA.

MATERIALS AND METHODS

Reagents. Hespan (6.2% hetastach in 0.9% NaCl) was obtained from Du Pont Pharmaceuticals. Lymphocyte separation medium was purchased from Organon Teknika. Catalytic subunit of PKA (from bovine heart, 40 units/μg protein), GTPγS, and triethanolamine were obtained from Sigma. [^{3}H]1-*O*-alkyl-lysophosphatidylcholine (192 Ci/mmol) were from Amersham Corp. [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from NEN. Silica gel 60 TLC plates were acquired from EM science. Recombinant RhoGDI was prepared from *E. coli* expressing human RhoGDI as described (15). Anti-RhoA was mouse anti-peptide monoclonal antibodies obtained from Santa Cruz Biotechnology. All other reagents and solvents used were of highest quality available commercially.

Preparation of neutrophil plasma membrane and cytosol. Human neutrophils were isolated from healthy donors and purified according to Bowman *et al.* (21) after informed consent was obtained. The cells were labeled with [^{3}H]alkyl-lysophosphatidylcholine (1.5 μCi/2 $\times$ 10^7 cells) for 90 min at 37°C. The [^{3}H]-labeled cells were resuspended in triethanolamine buffer (25 mM triethanolamine, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 3 mM NaCl, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptine, 1 mM PMSF). The cells were disrupted by nitrogen cavitation (pressurized at 450 psi for 20 min at 4°C). Plasma membranes and cytosolic fraction were isolated as described previously (15).

Assay of PLD with [^{3}H]-labeled membrane and lipid extraction. PLD assays were carried out according to Olson *et al.* (9). [^{3}H]Alkyl-lysophosphatidylcholine labeled plasma membrane (25 μg) and cytosol (50 μg) were preincubated with or without cPKA and ATP in triethanolamine buffer containing 1 μM okadaic acid for 10 min at 37°C. Incubations were carried out in the presence of 100 μM GTPγS, 1 μM $CaCl_2$, and 1.6% ethanol for 20 min. The reactions were terminated by transferring contents to glass tubes containing chloroform and methanol (1:2). Lipids were extracted and spotted on TLC plates (silica gel 60), which were developed with chloroform: methanol:acetic acid (90:10:10 by volume). Radioactivity was quantified using a Bioscan system 200 imaging scanner (Bioscan Inc.). Phosphatidylethanol (PEth) formed by transphosphatidylation is expressed as the percentage of total counts.

Extraction of membranous RhoA with cytosolic fraction and RhoGDI. Neutrophil plasma membranes (400 μg) were preincubated with cPKA and ATP in triethanolamine buffer for 10 min at 37°C and mixed with cytosol (800 μg) or 1 μM RhoGDI. After incubation for 20 min, plasma membranes and cytosol were reisolated by density gradient centrifugation (30%/50% sucrose) at 55,000 rpm for 20 min.

In vitro phosphorylation by PKA and immunoprecipitation of RhoA. Phosphorylation of plasma membrane protein (400 μg) with cPKA *in vitro* was carried out for 10 min at 37°C in a 100 μl final volume of phosphorylation buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptine, 1 mM PMSF, 0.1 mM cold ATP and 2 μCi [γ-^{32}P]ATP). 10 μM RhoGDI was added to the reaction mixture and further incubated for 20 min at 37°C. Plasma membranes and supernatant were obtained using a sucrose gradient. The supernatants used for immunoprecipitation were obtained after mixing PKA and ATP-pretreated plasma membrane with 10 μM RhoGDI and 1% Triton X-100. The samples were precleared with protein A–agarose for 1 h at 4°C and were incubated with 10 μl mouse anti-RhoA antibody for 1 h at 25°C. The immune complex was then transferred to 50 μl of 1:1 slurry of protein A–agarose beads and mixed for 1 h. The beads were pelleted and washed six times with phosphate buffered saline containing 1% Triton X-100. Samples were boiled in SDS–PAGE sample buffer and subjected to SDS–PAGE and autoradiography.

Immunochemical method. Western blotting was carried out by standard methods using alkaline phosphatase-conjugated second antibody. Mouse anti-RhoA antibody was used at dilution of 1:1000.

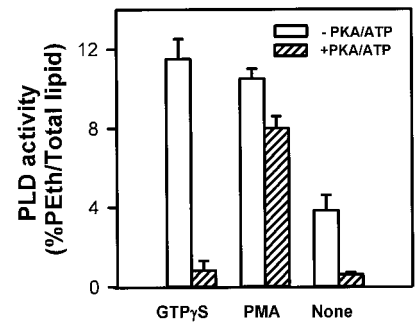

FIG. 1. Inhibition of GTPγS-activatable PLD in a cell-free system consisting of neutrophil plasma membrane and cytosol by PKA. Isolated plasma membranes (25 μg) and dialyzed cytosol (50 μg) were preincubated with or without 100 units cPKA or 1 mM ATP in triethanolamine buffer containing 1 μM $CaCl_2$ and 1.6% ethanol for 10 min at 37°C. Reaction was initiated by addition of 100 μM GTPγS, 100 nM PMA and 1 mM ATPγS, or nothing. After 20 min, the incubation was terminated and [^{3}H]phosphatidylethanol was quantified as described under Materials and Methods. Each value is the mean and standard error of three to four independent experiments.

RESULTS

cAMP-increasing agents have been previously shown to inhibit PLD activity in fMLP-stimulated intact neutrophils (17, 18). We did not observe inhibition of GTPγS-stimulated PLD activity when plasma membranes and cytosol were isolated from cells which had been treated with dibutyryl cAMP, prostaglandin E_2, and theophylline (data not shown). We therefore measured PLD activity in the cell-free system consisting of plasma membranes and cytosol in the presence of cPKA and ATP. GTPγS-stimulated PLD activity was inhibited up to about 90% by 100 units cPKA and 1 mM ATP (Fig. 1). cPKA inhibited activity to less than the basal PLD activity. PMA and ATPγS-stimulated activity was not significantly affected by cPKA. cPKA and ATP both caused a concentration-dependent inhibition of GTPγS-stimulated PLD activity (Fig. 2). GTPγS-stimulated PLD activity was slightly decreased with 0.5 mM ATP in the absence of cPKA but almost com-

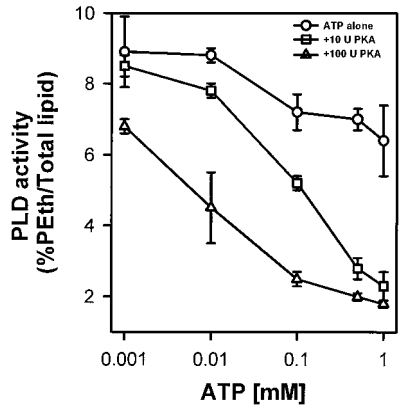

FIG. 2. Inhibition of GTPγS- stimulated PLD activity by increasing concentrations of PKA and ATP. Plasma membranes (25 μg) and dialyzed cytosol (50 μg) were first preincubated with increasing concentrations of ATP for 10 min at 37°C in the presence or absence of cPKA and then incubated for 20 min with 1 μM $CaCl_2$, 1.6% ethanol, and 100 μM GTPγS. PLD activity was determined as described under Materials and Methods. Error bars represent the range of two to three determinations for each point.

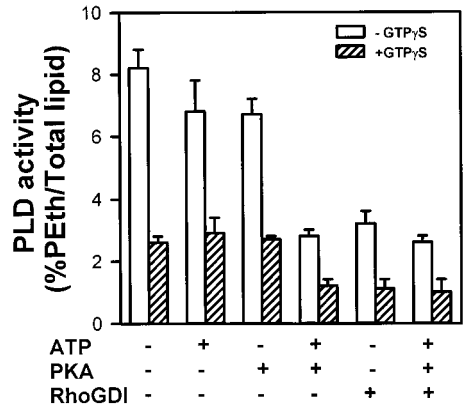

FIG. 3. Effect of PKA on the plasma membrane fraction in the inhibition of PLD activation. Plasma membranes (400 μg) were pretreated with 1 mM ATP, 100 units cPKA, and 10 μM RhoGDI and re-isolated using sucrose gradients as described under Materials and Methods. PLD stimulating activity was measured in the presence of pretreated plasma membranes (25 μg), untreated cytosol (50 μg), and GTPγS (100 μM). The data shown are representative of three independent experiments with similar results. Error bars represent the range of two to three determinations for each point.

pletely inhibited in the presence of 10 units cPKA. Concentrations of ATP as low as 10 or 100 μM in the presence of 100 units cPKA sharply decreased the PLD activity. In contrast to the inhibition of the activity with ATP, neither adenyl-5′-yl imidodiphosphate (App[NH]p), ADP, AMP, UTP, nor CTP (at 1 mM) inhibited PLD activity in the presence or absence of cPKA (data not shown).

Most PLD enzymes reside in plasma membrane although cytosolic PLD has been reported in HL-60 cells (8, 22). Regulatory factors in the cytosol may be translocated to interact with PLD on the plasma membrane. To investigate whether the target of PKA in the inhibition of PLD activity resides on the plasma membrane, plasma membranes were pretreated with cPKA and/or ATP, and re-isolated using sucrose gradients. Upon reconstitution of these cPKA and ATP-pretreated plasma membranes with naive cytosol, we observed an 80% reduction in GTPγS-stimulated PLD activity (Fig. 3). Pretreatment of plasma membranes with ATP or PKA alone did not inhibit PLD activity. The magnitude of inhibition by PKA was also identical to that elicited by RhoGDI. RhoGDI has been shown previously to inhibit PLD activity in a cell-free system of human neutrophils (13). Interestingly, the PLD activity was not completely inhibited by the combination of PKA and RhoGDI.

Plasma membrane components known to be associated with the regulation of PLD are RhoA and PLD itself (15). It has been reported that RhoA in cytotoxic lymphocytes is phosphorylated by PKA and that the phosphorylated form of RhoA is more easily dissociated from plasma membrane by cytosolic RhoGDI (23). Therefore, we measured the amount of RhoA in plasma membrane to examine the effect of PKA on RhoA. We did not notice changes in the level of immunodetectable RhoA in plasma membranes treated with PKA or ATP alone. However, as shown in Fig. 4, the addition of cytosol significantly increased dissociation of RhoA from the PKA-pretreated plasma membrane. ATP alone increased translocation of cytosolic RhoA to plasma membrane when both plasma membrane and cytosolic fractions were simultaneously incubated with ATP (data not shown). These results suggest that the target of PKA may be RhoA in plasma membrane.

To investigate whether PKA phosphorylates RhoA in the plasma membrane of human neutrophils, *in vitro* phosphorylation assays and immunoprecipitations of RhoA were performed. A phosphorylated band was observed at the position with an approximate molecular weight 20 kDa (Fig. 5A, lane 1) and the phosphorylated protein(s) could be extracted with RhoGDI (Fig. 5A, lane 2). As shown in Fig. 5A, lane 3, a 20-kDa radioactive band was still present in RhoGDI-treated

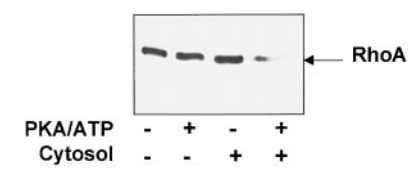

FIG. 4. Dissociation of RhoA from PKA-treated plasma membrane by the addition of cytosolic fractions. Plasma membranes (1 mg/ml) were incubated for 10 min at 37°C with buffer (lane 1), 1 mM ATP and 100 units cPKA (lane 2), 2 mg/ml cytosol (lane 3), and cytosol in the presence of 100 units cPKA and 1 mM ATP (lane 4). Membrane fractions were isolated from the middle layer of sucrose gradient (30%/50%). Proteins (15 μg) were immunoblotted with anti-RhoA antibody. Results are representative of two experiments.

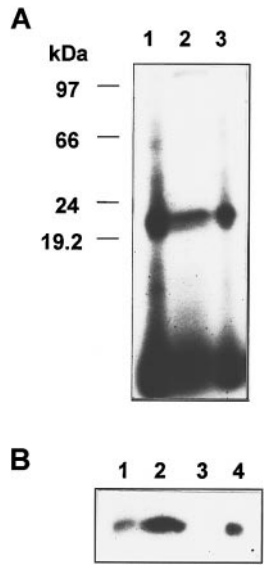

FIG. 5. *In vitro* phosphorylation of membranous RhoA by PKA. In A, PKA- phosphorylated proteins were extracted with RhoGDI. Plasma membranes (400 μg) were preincubated with 100 units cPKA, 0.1 mM cold ATP, and 2 μCi [γ-^{32}P]ATP in triethanolamine buffer (100 μl) for 10 min and further incubated with 10 μM RhoGDI for 20 min. PKA-treated plasma membrane is shown in lane 1. Supernatant (lane 2) and plasma membrane (lane 3) were reisolated after addition of RhoGDI and proteins were separated by SDS–PAGE followed by autoradiography. In the experiment of B, PKA-phosphorylated protein was immunoprecipitated with anti-RhoA antibody. Lane 1 shows radioactive band in PKA-treated plasma membrane as in A, lane 1. Plasma membranes were then treated with 1% Triton X-100 in addition to RhoGDI and supernatant was prepared (lane 2). The supernatant fractions were immunoprecipitated with anti-RhoA antibody and protein A–agarose. The supernatant fraction (lane 3) and beads (lane 4) obtained by immunoprecipitation were analyzed by SDS–PAGE and autoradiography. Similar results were reproduced in two additional experiments.

plasma membrane. Rap1A, a ras-related G protein, has been identified as a target for PKA in neutrophils (24). It has been reported that Rho proteins in the plasma membrane can be dissociated with RhoGDI and only RhoA among the Rho proteins is presently known to be phosphorylated by PKA (23, 25). When the supernatant containing Rho proteins extracted from the plasma membrane was immunoprecipitated with anti-RhoA antibody, a phosphorylated band was observed (Fig. 5B, lane 4). The addition of 1% Triton-X 100 to the incubation mixtures of the immunoprecipitation assay facilitated RhoA extraction from the plasma membrane. This observation in a neutrophil cell-free system is consistent with previous results in cytotoxic lymphocytes (23).

A suboptimal concentration of RhoGDI (1 μM) partially extracted RhoA from plasma membranes (Fig. 6). As shown in Fig. 6 more RhoA was extracted by treatment with both RhoGDI and cPKA rather than RhoGDI alone, suggesting that phosphorylated RhoA is more easily extracted from the plasma membrane by RhoGDI than the unphosphorylated form. However, RhoA in PKA-treated plasma membranes was not completely dissociated with RhoGDI. This result is consistent with the findings of activity assay in Fig. 3. The addition of GTPγS in preincubation mixture had no effect on the extraction of RhoA in this experiment.

DISCUSSION

Previous studies have raised several questions regarding the mechanism and target of cAMP on down-regulation of PLD in neutrophils. Because of the dual localization of PLD isoforms (membrane and cytosolic) and the growing cast of activator proteins, the regulation of PLD presents a complex picture. When intact cells were pretreated with dibutyryl cAMP, PGE_2, and theophylline, no inhibition of GTPγS-stimulated PLD activity was subsequently observed when PLD activity was tested in the cell-free system. We speculated that the effect may be transitory and any initial inhibition of PLD by cAMP increasing agents may be lost when cytosol and plasma membrane fractions were isolated and the PLD activity examined. We discovered that PLD activity was inhibited by the catalytic subunit of PKA in a cell-free system and this has enabled us to investigate the target and mechanism of inhibition by PKA.

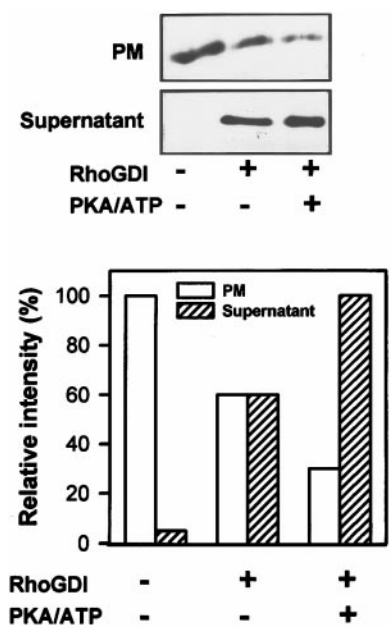

FIG. 6. Dissociation of RhoA by recombinant RhoGDI and PKA from plasma membrane. Plasma membrane (1 mg/ml) was incubated for 10 min at 37°C with buffer, 1 μM RhoGDI, or RhoGDI in the presence of 100 unit cPKA and 1 mM ATP. Membrane and supernatant fractions were reisolated under Materials and Methods. Plasma membranes (15 μg) and supernatant (15 μl) were resolved by SDS–PAGE and were immunoblotted with anti-RhoA antibody. Results are representative of four experiments. Lower panel shows densitometric values of the bands in upper panel.

Our present studies, showing that addition of cPKA in reaction mixture of membrane and whole cytosol inhibited GTPγS-stimulated but not PMA-stimulated PLD activity (Fig. 1), are in agreement with the findings that cAMP did not inhibit PMA-stimulated PLD activity in intact cells (6, 7). These data suggest that the target of PKA inhibition is one of the regulators of the enzyme rather than the enzyme itself. However, changes in the level of cAMP probably affect a variety of signaling pathways in intact neutrophils.

Another group has shown that activation of PLD by GTPγS was potentiated with ATP in a cell-free system from U937 cells (26). In our experiments, addition of ATP alone in the micromolar to millimolar range inhibited GTPγS-stimulated PLD activity in a reconstitution of membrane with cytosol. ATP has been shown to differentially affect PLD activation probably due to differences in assay conditions, regulatory factor(s) involved in the activation process, or the PLD isoforms in different cell type (16, 27–29). Consistent with our results, PLD activity in permeabilized human neutrophils was decreased when ATP was added but was enhanced by the nonspecific kinase inhibitor, staurosporine, suggesting a kinase(s) downregulates PLD activity in human neutrophils (29). Staurosporine also enhanced GTPγS-stimulated PLD activity in a cell-free system and fMLP-induced PLD activity in intact cells.[2]

PLD activity was reduced when cPKA and ATP pretreated-plasma membranes were reconstituted with naive cytosol. These results imply that membrane-associated PLD itself or regulatory factor(s) on the plasma membrane are the target of inhibition by PKA. Our previous studies showed that RhoA is a membrane activator of neutrophil PLD (15). Thus, we focused on the Rho subfamily of small GTP-binding proteins. Recently, it was reported that phosphorylation by PKA of GTP-bound RhoA enhanced the dissociation of RhoA from the plasma membrane to the cytosol in cytotoxic lymphocytes (23). Phosphorylation may affect the interaction of RhoA with PLD, as is the case for decreased binding of PKA-phosphorylated RhoA to RhoA-associated serine/threonine kinase (ROKα) (30) or decreased modulation of Rap1A association with cysteine rich region of its effector Raf1 by phosphorylation (31). Our current studies demonstrate that the membrane bound RhoA of human neutrophils is phosphorylated by PKA and is more readily dissociated from plasma membrane by recombinant RhoGDI as well as cytosol. This suggests that the dissociation of RhoA may be responsible for the reduced PLD activity. However, we can not rule out the possible involvement of other factor(s).

PKA and ATP treatment inhibited PLD activity when plasma membranes and cytosol were preincubated and stimulated with GTPγS (Fig. 3). However, the addition of RhoGDI to the PKA and ATP pretreated plasma membrane did not completely inhibit the GTPγS stimulated PLD activity. This discrepancy may be due to RhoGDI-bound RhoA in cytosolic fraction.

It is possible that the PLD is directly phosphorylated and affected by PKA. Unfortunately, PLD in human neutrophils has not been purified or identified definitively. Neutrophil PLD has some similarities to PLD1 because both are activated with RhoA, ARF, and PKC (32). When we investigated the effect of cPKA on PLD1 activity stimulated by ARF1 and GTPγS, cPKA had no significant inhibitory effect on PLD activity even though affinity purified PLD1 was phosphorylated *in vitro* by cPKA and [γ-^{32}P]ATP (data not shown). Although PLD in neutrophils has some similarities to PLD1, the results with the purified PLD differ from those observed in the neutrophil cell free system. Thus, it is not clear that showing phosphorylation of PLD1 by PKA is relevant to the neutrophil system.

It is also possible that PKA may affect the interaction between the enzyme and its regulators through phosphorylation of RhoA or the PLD enzyme. Frohman *et al.* (33) reported that the site of interaction of PLD1 with Rho proteins is in the carboxyl terminus and this region is well conserved between PLD1 and PLD2. They also showed that the amino terminus of PLD1 is required for PLD1 to respond to PKC. Our finding that PMA-stimulated PLD activity was not inhibited by PKA suggests that the interaction between PKC and PLD is not affected by PKA or that PKC acts upstream of RhoA.

The cell-free system provides a useful model to investigate the effect(s) of PKA phosphorylation on the interaction between the PLD enzyme and its regulators. Further studies will be required to understand the complex cross- talk between PKA and PLD in the signaling pathways of neutrophil activation.

ACKNOWLEDGMENT

The authors acknowledge the financial support of the Korea Research Foundation made in the program year of 1997.

REFERENCES

1. Billah, M. M., and Anthes, J. C. (1990) *Biochem. J.* **269,** 281–291.
2. Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W., and Siegel, M. I. (1989) *J. Biol. Chem.* **264,** 17069–17077.
3. Perry, D. K., Hand, W. L., Edmondson, D. E., and Lambeth, J. D. (1992) *J. Immunol.* **149,** 2749–2758.
4. Bokoch, G. M. (1995) *Blood* **86,** 1649–1660.
5. Exton, J. H. (1997) *Physiol. Rev.* **77,** 303–320.
6. Cockcroft, S. (1992) *Biochim. Biophys. Acta* **1113,** 135–160.
7. Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212,** 26–42.
8. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem.* **66,** 475–509.

[2] J.-Y. Kwak, unpublished observation.

9. Olson, S. C., Bowman, E. P., and Lambeth, J. D. (1991) *J. Biol. Chem.* **266,** 17236–17242.
10. Brown, H., Gutowski, S., Moomaw, C., Slaughter, C., and Sternweis, P. (1993) *Cell* **75,** 1137–1144.
11. Cockcroft, S., Thomas, G., Fensom, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N., Truong, O., and Husuan, J. (1994) *Science* **263,** 523–526.
12. Lambeth, J. D., Kwak, J.-Y., Bowman, E., Perry, D., Uhlinger, D., and Lopez, I. (1995) *J. Biol. Chem.* **270,** 2431–2434.
13. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) *J. Biol. Chem.* **268,** 21509–21512.
14. Malcolm, K. C., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1994) *J. Biol. Chem.* **269,** 25951–25954.
15. Kwak, J.-Y., Lopez, I., Uhlinger, D. J., Ryu, S. H., and Lambeth, J. D. (1995) *J. Biol. Chem.* **270,** 27093–27098.
16. Lopez, I., Burns, D. J., and Lambeth, J. D. (1995) *J. Biol. Chem.* **270,** 19465–19472.
17. Agwu, D. E., McCall, C. E., and McPhail, L. C. (1991) *J. Immunol.* **146,** 3895–3903.
18. Tyagi, S. R., Olson, S. C., Burnham, D. N., and Lambeth, J. D. (1991) *J. Biol. Chem.* **266,** 3498–3504.
19. De Togni, P., Cabrini, G., and Di Virgilio, F. (1984) *Biochem. J.* **224,** 629–635.
20. Sedgwick, J. B., Berube, M. L., and Zurier, R. B. (1985) *Clin. Immunol. Immunopathol.* **34,** 205–215.
21. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1995) *Methods Enzymol.* **257,** 246–256.
22. Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1995) *J. Biol. Chem.* **270,** 8466–8473.
23. Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996) *EMBO J.* **15,** 510–519.
24. Quilliam, L. A., Mueller, H., Bohl, B. P., Prossnitz, V., Sklar, L. A., Der, C. J., and Bokoch, G. M. (1991) *J. Immunol.* **147,** 1628–1635.
25. Ueda, T., Kikuchi A., Ohga N., Yamamoto, J., and Takai, Y. (1990) *J. Biol. Chem.* **265,** 9373–9380.
26. Kusner, D. J., Schomisch, S. J., and Dubyak, G. R. (1993) *J. Biol. Chem.* **268,** 19973–19982.
27. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) *J. Biol. Chem.* **270,** 5130–5135.
28. Steed, P. M., Nagar, S., and Wennogle, L. P. (1996) *Biochemistry* **35,** 5229–5237.
29. Bauldry, S. A., and Wooten, R. E. (1998) *FASEB J.* **12,** A1384.
30. Dong, J.-M., Leung, T., Manser, E., and Lim, L. (1998) *J. Biol. Chem.* **273,** 22554–22562.
31. Hu, C.-D., Kariya K.-I., Okada, T., Qi X., Song, C., and Kataoka T. (1999) *J. Biol. Chem.* **274,** 48–51.
32. 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.
33. Sung, T. C., Roper, R., Zang, Y., Rudge, S. A., Temel, R., Hammond, S. M., Morris, A. J., Moss, B., Engelbrecht, J., and Frohman, M. A. (1997) *EMBO J.* **16,** 4519–4530.