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ACTIVATION OF MEMBRANE-BOUND PHOSPHOLIPASE D BY PROTEIN KINASE C IN HL60 CELLS : SYNERGISTIC ACTION OF A SMALL GTP-BINDING PROTEIN RhoA

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SUMMARY : Regulation of phospholipase D (PLD) activation by protein kinase C (PKC) was studied in membranes isolated from human promyelocytic leukemia HL60 cells. The activation of membrane-bound PLD by PKC partially purified from rat brain was most effectively induced with phorbol 12-myristate 13-acetate (PMA) and Ca^{2+} (1 μM) which caused translocation of PKC to membranes. Ro31-8425, a potent inhibitor of PKC, suppressed the catalytic activity of PKC in a concentration-dependent manner, with complete inhibition at 5 μM. However, the PKC-mediated PLD activation was not affected by Ro31-8425. It was thus suggested that membrane-bound PLD of HL60 cells was activated by PKC translocation but probably via a phosphorylation-independent mechanism. Furthermore, addition by guanosine 5'-3-*O*-(thio)triphosphate (GTPγS) potentiated the PKC-mediated PLD activation and this potentiating effect was abolished by Rho GTPase dissociation inhibitor (RhoGDI). The suppressed PLD activation by RhoGDI was completely restored by addition of recombinant RhoA. These results indicate that the PKC-mediated PLD activation can be synergistically potentiated by RhoA in HL60 membranes.

Phospholipase D (PLD) has been known to play an important role in signal transduction of a variety of cells. PLD hydrolyzes phosphatidylcholine (PC) to generate phosphatidic acid (PA), which is subsequently metabolized via PA phosphohydrolase to form diacylglycerol (DG) (1). From studies in cell-free system and permeabilized cells, it has been known that PLD activity can be regulated by several factors, such as GTP-binding proteins, protein kinases and Ca^{2+} (1).

Protein kinase C (PKC) has been implicated in the course of PLD activation in various cell types (2). Evidence that PKC upregulates PLD activity is supported by observations that PKC inhibitors or PKC down-regulation by long-term exposure to phorbol ester prevent the increase of PLD activity (1) and also that PKC overexpression in cells enhances PLD activity (3-5). Furthermore, a recent study demonstrated that a purified PKC directly activated PLD in CCL39

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Abbreviations : PLD, phospholipase D; PBut, phosphatidylbutanol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; GTPγS, guanosine 5'-3-*O*-(thio)triphosphate; PC, phosphatidylcholine; ARF, ADP-ribosylation factor; GDI, GTPase dissociation inhibitor.

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fibroblasts membranes (6, 7). Despite such abundant data, the molecular mechanisms of PLD activation by PKC remain unknown. It has also been suggested that PLD could be activated by a phosphorylation-independent mechanism of PKC (6, 8-10).

On the other hand, GTPγS-induced PLD activity indicates involvement of GTP-binding protein. Recent studies have demonstrated that two small GTP-binding proteins, ADP-ribosylation factor (ARF) (11, 12) and RhoA (13, 14) activate membrane-bound PLD in several types of cells. Furthermore, several reports suggested that GTP-binding proteins synergized with PKC to cause activation of PLD (9, 15, 16). The precise relationship between ARF / RhoA and PKC, however, has not yet been disclosed in reconstitution system.

In the present study, we have examined the activation mechanism of PLD by PKC in membranes isolated from human promyelocytic leukemia HL60 cells, and have demonstrated that the membrane-bound PLD of HL60 cells was activated by PKC translocated to membranes and that the PKC-induced PLD activation was synergistically potentiated by RhoA.

MATERIALS AND METHODS

Materials : Antibodies against PKC isozymes (α, β1, β2, γ, δ, ε) and small GTP-binding proteins (RhoA, Rac1/Rac2, Cdc42Hs) were purchased from Santa Cruz Biotechnology, Inc. Recombinant RhoA and GST-RhoGDI were kindly supplied by Dr. Y. Takai (Osaka University).

Cell culture and preparation of plasma membranes : HL60 cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in humidified atmosphere of 5 % CO_2 at 37 °C. Cells were labeled with [^{3}H] oleic acid (0.5 μCi/ml) for 12-15 hr. About 65 % of total lipid radioactivity was incorporated into phosphatidylcholine (PC). The labeled cells were washed twice with phosphate-buffered saline and resuspended in buffer A (25 mM HEPES pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 0.5 mM MgATP, 1 mM EGTA, 5 mM dithiothreitol (DTT), 0.5 mM phenylmethyl-sulfonylfluoride (PMSF), and 100 μg/ml E-64). Cells were then disrupted in N_2 cavitation (600 p.s.i. at 4 °C for 30 min). After unbroken cells were removed by centrifugation at 900 x *g* for 5 min, membrane were collected by centrifugation at 100,000 x *g* for 60 min and resuspended in buffer A.

Assay of PLD activity in membranes : Isolated HL60 membranes (50 μg protein/assay) were resuspended in buffer A containing $CaCl_2$ to give a final free Ca^{2+} concentration of 1μM with or without partially purified PKC (total reaction 100 μl) and were stimulated with 100 nM PMA or 10 μM GTPγS at 37 °C for 15 min in the presence of butanol (0.3 %, v/v). Lipids were extracted according to the method of Bligh and Dyer (17) and separated as described previously (18, 19). PLD activity was measured by production of [^{3}H]phosphatidylbutanol (PBut).

Purification of PKC from rat brain cytosol : Rat brain PKC was purified as described previously (20). After chromatographies, fractions were subjected to the assay for PKC activity and Western blotting analysis using specific antibodies against PKC α, β1, β2, δ, ε isozymes and small GTP-binding proteins (RhoA, Rac1/Rac2, Cdc42Hs).

Assay of protein kinase C activity : The PKC activity was assayed as described previously (21) using by myelin basic protein (MBP) (22). 1 unit PKC was expressed as 1 pmol [γ-^{32}P] ATP incorporated in MBP /5 min.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis : SDS-PAGE was performed on 8 or 12 % polyacrylamide gels as described by Laemmli (23). Western blotting analysis using specific antibodies against PKC or small GTP-binding proteins was conducted as previously described (24).

RESULTS AND DISCUSSION

Activation of HL60 membrane-associated PLD by PKC purified from rat brain

To examine the mechanisms of PKC-mediated PLD activation, we have used isolated HL60

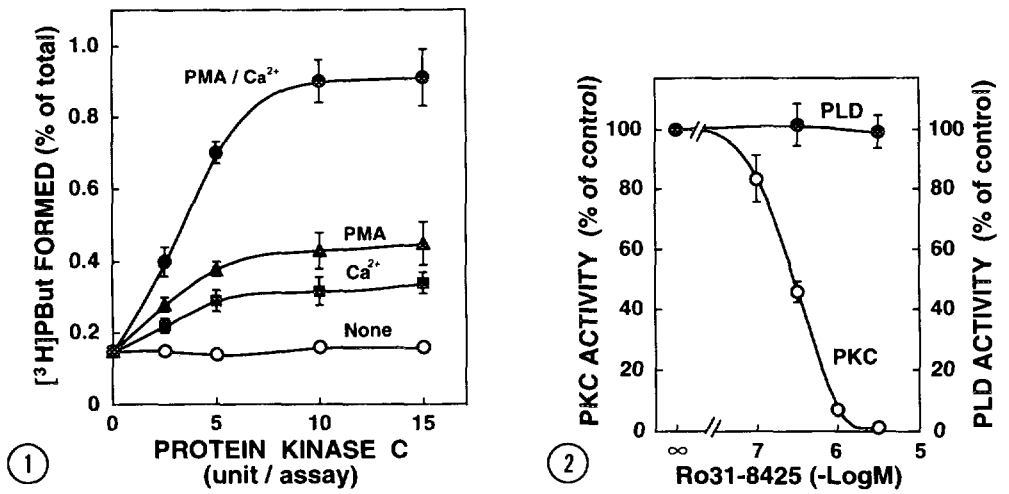

Fig. 1. Activation of PLD in HL60 membranes by partially purified PKC fraction. The HL60 membranes (50 μg protein) and partially purified PKC fraction at the indicated concentrations were incubated without (○) or with 1 μM Ca^{2+} (■), 100 nM PMA (▲), or both 1 μM Ca^{2+} and 100 nM PMA (●) at 37 °C for 15 min. Measurement of PLD activity was performed as described in Materials and Methods. Data represent the mean ± S.D. of two different experiments carried out in duplicate.

Fig. 2. Effect of Ro31-8425 on the PKC-mediated PLD activation in HL60 membranes. Partially purified PKC fraction (7.5 units/assay) was incubated at 37°C for 10 min with PKC inhibitor, Ro31-8425,at the indicated concentrations and then PKC activity (○) was measured by incorporation of ^{32}P into MBP as substrate for 15 min. PLD activity (●) was measured by formation of [³H]PBut for 15 min in the presence of HL60 membranes (50 μg protein). The activation obtained in the absence of Ro31-8425 was expressed as 100 %. Data represent the mean ± S.D. of two different experiments carried out in duplicate.

membranes and a partially purified rat brain PKC fraction. Several studies have indicated that in HL60 cells PLD is regulated by various factors such as PKC (8, 15), small GTP-binding proteins (15, 25-31) and Ca^{2+} (25-29). The PKC used in the present study was partially purified from rat brain cytosol by chromatographies on anion exchange (Mono Q) and Superose 12 columns. The partially purified PKC fraction contained PKC-α, β1, β2 and lesser amount of PKC-ε, but did not contain RhoA, Rac1/Rac2, Cdc42Hs when examined by Western blotting using specific antibodies (data not shown). In addition, PLD activity was not present in this PKC fraction when measured with PE/PIP_2/PC (16:1.4:1) as substrate.

As shown in Fig. 1, the PLD activity of HL60 membranes was stimulated by the PKC fraction in a concentration-dependent manner. The PKC fraction alone had no effect on PLD activation, whereas a 2-3 fold increase in PKC-mediated PLD activation was observed when Ca^{2+} (1 μM) or/and PMA (100 nM) was present in the reaction mixture. PMA and Ca^{2+} exerted a synergistic augmentation of PKC-mediated PLD activation. An inactive phorbol analogue, 4α-PMA was ineffective. Incubation of the HL60 membranes with the PKC fraction in the presence of Ca^{2+} and PMA caused translocation of PKC-α, β1, and β2 isozymes to membranes as inferred by Western blotting (data not shown). Furthermore, the recombinant PKC-α also stimulated 4-5

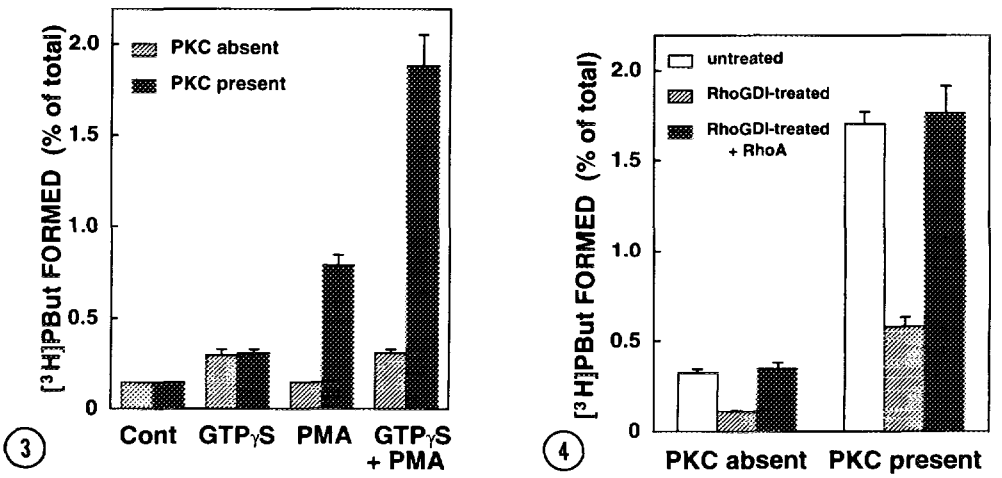

Fig. 3. Synergistic action by GTPγS of the PKC-mediated PLD activation in HL60 membranes. The HL60 membranes (50 μg protein) were stimulated with 100 nM PMA or 10 μM GTPγS or both PMA and GTPγS for 15 min in the absence or presence of partially purified PKC fraction (7.5 units/assay). Measurement of PLD activity was performed as described in Materials and Methods. Data represent the mean ± S.D. of two different experiments carried out in duplicate.

Fig. 4. Effect of RhoGDI and RhoA on the PKC-mediated PLD activation in HL60 membranes. The HL60 membranes (50 μg protein) were incubated with or without 7 μM GST-RhoGDI at 4 °C for 30 min, and then washed once in buffer A. The washed membranes were stimulated with 10 μM GTPγS and 70 nM recombinant RhoA in the absence of PKC or with 10 μM GTPγS, recombinant RhoA and 100 nM PMA in the presence of PKC (7.5 units/assay) for 15 min. Measurement of PLD activity was performed as described in Materials and Methods. Data represent the mean ± S.D. of two different experiments carried out in duplicate.

fold the HL60 membrane-bound PLD activity (data not shown). These results suggested that at least PKC-α may play a role in PKC-mediated PLD activation in HL60 membranes.

It was shown that a potent PKC inhibitor, Ro31-8425, suppressed the PMA-induced PKC activation in a concentration-dependent manner (Fig.2). 5 μM Ro31-8425 completely inhibited the catalytic activity of PKC as measured by ATP-dependent phosphorylation of MBP. The inhibitory potency was not changed in the presence of 0.5 mM MgATP in the reaction mixture for PLD activation. Although PKC activity was markedly reduced by the inhibitor, it had no effect on PLD activation by PKC in HL60 membranes (Fig.2). In addition, Ro31-8425 did not affect the translocation of PKC isozymes (α, β1, β2) when examined by Western blotting (data not shown). These results indicated that the HL60 membrane PLD activation by PKC may not be associated with ATP-dependent phosphorylation. Billah *et al.* (8) have reported that PMA-induced protein phosphorylation by PKC is not involved in PLD activation in HL60 cells. Furthermore, studies in other cell types have also suggested a PKC-mediated PLD activation by a phosphorylation-independent mechanism (9, 10). Conricode *et al.* (6) also suggested that PKC activates PLD by an allosteric mechanism without ATP-dependent phosphorylation.

Effect of GTPγS on PKC-mediated PLD activation HL60 membranes

Several studies have demonstrated evidences which suggest that small GTP-binding proteins synergize with PKC in PLD activation (9, 15, 16). We examined effects of GTPγS and

PMA on PLD activation in HL60 membranes in the presence or absence of PKC. As shown in Fig.3, the membrane PLD was stimulated 2 fold by GTPγS (10 μM) with or without PKC. On the other hand, the PMA-induced PLD activation in the presence of PKC was remarkably potentiated by addition of GTPγS. Therefore, it was considered that PKC must be translocated to membranes for synergistic action with GTP-binding protein(s) in the membrane PLD activation.

Effects of RhoGDI and RhoA on PKC-mediated PLD activation in HL60 membranes

It has been reported that a membrane PLD was activated by small GTP-binding proteins such as RhoA and ARF (11-14). Since RhoA was present in HL60 membranes as inferred by its antibody, we have examined involvement of RhoA in PKC-mediated PLD activation. To this end, recombinant RhoGDI and RhoA were used. As shown in Fig.4, GTPγS-induced PLD activation in HL60 membranes in the absence of PKC was almost completely inhibited by addition of 7 μM RhoGDI, suggesting that GTPγS-induced PLD activation was responsible for small GTP-binding proteins Rho family. Furthermore, the suppressed GTPγS-induced PLD activation was fully restored to the initial level before RhoGDI treatment by addition of 70 nM RhoA, suggesting that the PLD of HL60 membranes was activated by RhoA.

The effects of RhoA and RhoGDI were also distinct in the presence of PKC (Fig.4). The synergistic activation of PKC-mediated PLD of HL60 membranes by GTPγS was markedly prevented by pretreatment with 7 μM RhoGDI. The RhoGDI treatment did not affect translocation to membranes of PKC when examined by Western blotting (data not shown). The repressed activity was completely restored to the control level by addition of 70 nM RhoA (Fig.4). The membrane translocation of PKC was unaffected by RhoA. These findings indicated that PKC translocated by PMA may play an important role in a synergistic activation of PLD with RhoA in HL60 membranes. From the results obtained here, it is tempting to speculate that RhoA exerts a synergistic action in the PKC-mediated activation of membrane PLD in HL60 cells.

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