Regulation of Phospholipase D2 Activity by Protein Kinase Cα*

Received for publication, October 7, 2003, and in revised form, February 27, 2004
Published, JBC Papers in Press, March 18, 2004, DOI 10.1074/jbc.M311033200

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It has been well documented that protein kinase C (PKC) plays an important role in regulation of phospholipase D (PLD) activity. Although PKC regulation of PLD1 activity has been studied extensively, the role of PKC in PLD2 regulation remains to be established. In the present study it was demonstrated that phorbol 12-myristate 13-acetate (PMA)-induced PLD2 activation and phosphorylation declined. In summary, the data demonstrated that as the phosphorylation of PLD2 increased, its activation and activation. Co-immunoprecipitation studies in vitro and in vivo, suggesting the possible regulation of PLD2 activity by PKC (19, 21, 22). Hence, the role of PKC in PLD2 activation remains to be defined.

Regarding the PKC regulation of PLD1 activity, both phosphorylation-dependent (23, 24) and -independent mechanisms (5, 25, 26) have been proposed. A protein-protein interaction rather than phosphorylation has been proposed as the main mechanism for PKCα to activate PLD1 (26). Compared with PLD1, only one group has explored the phosphorylation of PLD2 by PKC and its effect on activity (22). This group reported that PKCδ is involved in PMA-induced PLD2 phosphorylation and activation in PC12 cells (22), whereas another group reported that PLD2 activity is enhanced by dephosphorylation of Ser/Thr residues (27). Thus, the role of phosphorylation in the activation of PLD2 by PKC requires further work.

We report in the present study that PMA induces PLD2 activation via the involvement of PKCα and that PLD2 becomes phosphorylated on both Ser and Thr residues. In addition, we demonstrate PKCα and PLD2 interact in vivo and that the phosphorylation is not required for the activation of PLD2. Evidence is presented that phosphorylation more likely contributes to deactivation of PLD2.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylinositol (PC) to phosphatidic acid (PA), and choline (1). PA is an important “second messenger” in several physiological processes (2). PA can be further metabolized to diacylglycerol by PA phosphohydrolase or to lysophosphatidic acid by phospholipase A2. Diacylglycerol is a well known activator of protein kinase C (PKC), whereas lysophosphatidic acid mediates many important physiological functions via its G protein-coupled receptors (3). Thus, PLD influences many important intracellular events via producing these downstream products. PLD activity is regulated by many stimuli such as growth factors, cytokines, hormones, neurotransmitters, and other molecules involved in extracellular communication (1). PLD is thought to play an important role in secretion, membrane trafficking, cytoskeleton reorganization, and apoptosis (1). PLD1 and PLD2 are two isoforms of mammalian PLD that share about 50% amino acid similarity. However, their regulatory properties are quite different, e.g. that PLD1 has a low basal activity and is regulated by PKC and members of the Rho, adenosine diphosphate ribosylation factor, and Ras/Ral families of small G proteins (4–9). In contrast, PLD2 exhibits a high basal activity when assayed in vitro and shows little or no in vitro response to the activators of PLD1 (10–12). However, other regulators of PLD2 activity have also been found. These activators include phosphatidylinositol 4,5-bisphosphate (10), unsaturated fatty acid (17), caveolin (18), calcium (19), Gαq-11 activator (20), and adenosine diphosphate ribosylation factor (12). Although the regulation of PLD1 by PKC is universally accepted, it is controversial on whether or not PLD2 is also regulated by PKC. The initial report showed that PLD2 was largely refractory to PKC activation in vitro and in vivo (11). However, recent reports demonstrate that PMA can activate PLD2 in vivo, suggesting the possible regulation of PLD2 activity by PKC (19, 21, 22). Hence, the role of PKC in PLD2 activation remains to be defined.

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‡ The abbreviations used are: PLD, phospholipase D; PKC, protein kinase C; PA, phosphatidic acid; Bis, bisindolylmaleimide I; PMA, phorbol 12-myristate 13-acetate; PC, phosphatidylinositol; PtdBut, phosphatidylbutanol; KN, kinase negative.
**EXPERIMENTAL PROCEDURES**

**Materials**—4β-Phorbol 12-myristate 13-acetate, phosphatidylinositol 4,5-bisphosphate, bovine serum albumin, and Triton X-100 were from Sigma. Phosphatidylethanolamine, PC, and phosphatidylbutanol (PtdBut) standard were from Avanti Polar Lipids Corp. Dopalmityl[2-palmitoyl-9,10-3H]Ptd, [γ-32P]ATP, and [3H]myristic acid were from PerkinElmer Life Sciences. Protein G-agarose beads, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, Tris-glycine SDS-polyacrylamide gels, PdDNAs(1+), PdDNAs(1),HisA, -B, -C) vectors, anti-Xpress monoclonal antibody, and fetal bovine serum were from Invitrogen. The transfection reagent FuGENE 6 and the protease inhibitor mixture were from Roche Applied Science. COS-7 cells were from the American Type Culture Collection. Anti-phosphoserine and anti-phosphothreonine polyclonal antibodies were from Zymed Laboratories Inc. Anti-PKCβ rabbit antiserum was from Upstate. Anti-PCKα, -β, -γ, -δ, -ε, -η, -θ, and - μ monoclonal antibodies were from BD Transduction Laboratories. Ro-31-8220, bisindolylmaleimide I, G, GE6976, and rottlerin were from Calbiochem. Human recombinant PKCa was from Panvera.

**Cell Culture and Transfection**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum in humidified 10% CO2. Six-well plates were seeded with 2 × 106 cells per well, and 10-cm dishes were seeded with 8 × 106 cells and transfected with FuGENE 6 according to the manufacturer’s instructions.

**In Vivo PLD Assay**—After a 6-h transfection with FuGENE 6, COS-7 cells in 6-well plates were serum-starved (0.5% serum) in Dulbecco’s modified Eagle’s medium in the presence of 1 μCi/ml [3H]myristic acid. After overnight starvation, PLD activity was assayed as described (28). Briefly, cells were preincubated in 0.3% 1-butanol and then treated as indicated in the figure legends. Cells were then washed with ice-cold phosphate-buffered saline and stopped with methanol. Lipids were extracted, and the PtdBut product was resolved by thin layer chromatography. Bands co-migrating with a PtdBut standard were scraped and quantitated by liquid scintillation counting.

**Subcellular Fractionation**—COS-7 cells in 100-mm plates were harvested after transfection with PLD2 constructs and starved overnight as described above. After treatment with the appropriate reagents, the cells were washed and harvested with ice-cold phosphate-buffered saline. Cells were then centrifuged and resuspended in 500 μl of ice-cold lysis buffer (25 mM Hepes, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 0.4 mM CaCl2, 0.25 mM EDTA, 0.4 mM CaCl2, 0.1% Triton X-100) for 30 min and then resuspended in 500 μl of 1:1 slurry of protein G-agarose beads (Pansera). After washing twice with the lysis buffer and then resuspended in SDS sample buffer and analyzed by Western blotting.

**In Vitro Phosphorylation of PLD2 by PKCα**—Immunoprecipitated PLD2 from PLD2-overexpressing COS-7 cells was washed with phosphorylation buffer (30 mM Tris/HC1, pH 7.0, 6 mM MgCl2, 0.25 mM EGTA, 0.4 mM CaCl2, 0.1% Triton X-100) four times and resuspended in the phosphorylation buffer. Aliquots of beads containing immunoprecipitated PLD2 were then mixed with 0.12 μg ATP, 10 μCi of [γ-32P]ATP, 100 ng of PKCa, and 100 ng PMA. The mixture was incubated at 30 °C for 30 min and then electrophoresed through an 8% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane, and the membrane was exposed to a photographic film for autoradiography.

**RESULTS**

**PMA Activates PLD2 Activity**—As seen in Fig. 1, the transphospholipidation product of PLD action, PtdBut, increased as early as 1 min after PMA treatment of COS-7 cells and accumulated in a time-dependent manner. PtdBut levels reached a maximum at around 15 min after PMA treatment. The data indicated that PLD2 was rapidly activated by PMA and that the activity of PLD2 (formation of PtdBut) dropped to the basal level after 15 min, indicating a short-duration response. The data demonstrated that PLD2, like PLD1, is also activated by PMA when overexpressed in COS-7 cells.

**PKC Mediates PMA-induced PLD2 Activation**—Because it is well established that PMA activates conventional and novel PKC isozymes, we used PKC inhibitors to explore the involvement of PKC in the PLD2 activation. Fig. 2 shows the effects of the PKC inhibitors on both basal and PMA-stimulated PLD activity in vector- and PLD2-transfected COS-7 cells. As a control, the effects of the PKC inhibitors on PLD1 activity were also examined. General PKC inhibitors such as Ro-31-8220 and bisindolylmaleimide I (Bia) showed strong inhibition of PMA-induced activation of control (vector) PLD activity and of PLD1 and PLD2 activity (Fig. 2). However, the conventional PKC

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2 In the absence of PMA, Ro-31-8220 induced some activation of PLD1 and PLD2. This probably relates to its ability to activate PKCa as shown by the membrane translocation of this PKC isozyme (data not shown).
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Fig. 2. Effect of PKC inhibitors on basal or PMA-stimulated PLD activity in vector-, PLD1-, or PLD2-transfected COS-7 cells. Vector, rPLD1, or rPLD2 was overexpressed in COS-7 cells. Cells were preincubated with 5 μM Ro-31-8220, 5 μM Bis, 0.5 μM Gö 6976, 15 μM rottlerin, or vehicle (Me2SO) for 20 min, respectively. Then the cells were treated with vehicle or 100 nM PMA for 30 min in the presence of 0.3% 1-butanol. PLD activity was measured as described under “Experimental Procedures.” Mean ± S.E. values from at least five experiments are shown.

PLD2 Undergoes Serine and Threonine Phosphorylation after PMA Treatment—The possible Ser/Thr phosphorylation of PLD2 after PMA treatment was examined using anti-phospho-Ser and anti-phospho-Thr antibodies. As indicated in Fig. 3A, PLD2 was basally phosphorylated on Thr but not on Ser residues. Upon PMA treatment, PLD2 became phosphorylated on Ser and Thr in a time-dependent manner. However, the phosphorylation on Ser became detectable only after 5 min of PMA treatment. The increase of phosphorylation was further demonstrated by the slower migration of PLD2 on the gel upon PMA treatment for longer times, as indicated by the Western blotting with Xpress antibodies in Fig. 3A.

Next, the effect of the PKC inhibitors on PMA-induced PLD2 phosphorylation was determined. As indicated in Fig. 3B, Ro-31-8220 and Bis completely inhibited the phosphorylation induced on both residues by treatment with PMA. Gö 6976 also showed a significant inhibition, whereas rottlerin had no effect at all. These data demonstrate that PKC, possibly the conventional isoforms rather than PKCδ, are involved in the phosphorylation of PLD2 induced by PMA.

PKCa Mediates PMA-induced PLD2 Phosphorylation—To obtain further information about which PKC isoform mediates the phosphorylation, the expression of different PKC isoforms in COS-7 cells was examined by Western blotting. Fig. 4A shows that PKCa, PKCd, PKCe, PKCi, and PKCζ were detected, whereas PKCβ, PKCγ, PKCη, and PKCθ were not even though the antibodies were shown to be effective (data not shown). Among the isoforms, PKCa showed a complete translocation from cytosol to membrane fractions after PMA treatment. In sharp contrast to PKCa, PKCd and PKCe were located mainly in the membrane fraction and showed little or no translocation when stimulated. These data imply that PKCa is more likely than other PKC isoforms to be involved in PMA-induced PLD2 phosphorylation.

To further explore this issue, PKCa or PKCd was overexpressed together with PLD2 in COS-7 cells, and PMA-induced PLD2 phosphorylation was examined. As shown in Fig. 4B, co-expression of PKCa greatly enhanced PMA-induced PLD2 phosphorylation on both Ser and Thr residues. In contrast, co-expression of PKCd had minimal effect. The lower panel of Fig. 4B shows that PKCa and PKCd were well expressed. The data provide further support that PKCa, rather than PKCd, is involved in PMA-induced PLD2 phosphorylation. To test if PKCa can directly phosphorylate PLD2, in vitro phosphorylation of PLD2 by PKCa was conducted. As shown in Fig. 4C, both PLD1 and PLD2 were phosphorylated by PKCa in the presence of PMA, although the phosphorylation of PLD1 was greater.

Phosphorylation Is Not Required for PLD2 Activation by PKCa—The requirement of phosphorylation for PLD2 activation by PKCa is next explored. First, the dose response of PMA on PLD2 phosphorylation was compared with that for PMA on PLD2 activation. Fig. 5A shows that PMA induced PLD2 phosphorylation by a dose-dependent manner. The phosphorylation was detectable at a PMA concentration of 2.5 nM and higher. Fig. 5B shows the dose dependence for PMA activation of PLD2. In contrast to the phosphorylation pattern, activation was evident at PMA concentrations as low as 0.5 nM and was maximal at 5 nM. The difference between these two patterns indicates that activation of PLD2 is not dependent on its phosphorylation.

To further confirm that phosphorylation is not required for PKCa-mediated PLD2 activation, a kinase-dead mutant of PKCa (KN-PKCa) was tested for its effect on the activation of PLD2. As shown in Fig. 6A, wild type PKCa enhanced PLD2 activity when co-transfected with PLD2 in COS-7 cells. Surprisingly, the kinase-dead PKCa mutant displayed an even
stronger stimulation than wild type PKCα on basal and PLD2 activity. As a control, PKCδ and PKCe were also co-expressed with PLD2 and showed no significant stimulation, showing the specificity for PKCα activation of PLD2. Fig. 6B confirms that, although wild type PKCα enhanced both basal and PMA-induced PLD2 phosphorylation, the kinase-dead mutant was unable to phosphorylate PLD2. Fig. 6C further shows that in contrast to wild type, kinase-dead PKCα does not exhibit autophosphorylation after PMA treatment. These data strongly support the conclusion that phosphorylation is not required for PKCα to activate PLD2 activity.

PLD2 Binds to PKCα after PMA Treatment—Consistent with the fast activation of PLD2 by PKCα in vivo, PMA induced a rapid translocation of this PKC isoform from cytosol to the membrane fraction (Fig. 7A). Because PLD2 localizes to membranes (11, 29), this raises the possibility that the translocation of PKCα makes it more accessible to interact with PLD2. To explore this, the co-immunoprecipitation of PLD2 and PKCα was examined in COS-7 cells. Fig. 7B shows that there is a basal association between PLD2 and PKCα, and the binding is greatly enhanced by PMA treatment. Fig. 7C shows that, consistent with the results in Fig. 7B, PLD2 was present in PKCα immunoprecipitates and that PMA potentiated the association in a time-dependent manner. These data demonstrate that PLD2 can interact with PKCα. To examine whether the interaction between PLD2 and PKCα is specific, the presence of endogenous PKCα, PKCδ, or PKCe in PLD2 immunoprecipitates was determined. As shown in Fig. 7D, endogenous PKCα is associated with PLD2, and the association is increased after PMA treatment in a time-dependent manner. In contrast, no PKCδ or PKCe was detected in PLD2 immunoprecipitates before and after PMA treatment. These data therefore show that PKCα rather than PKCδ or PKCe mediates PMA-induced PLD2 activation. The effect of PKC inhibitors on the PLD2-
PKCα interaction was also examined. As shown in Fig. 7E, Ro-31-8220, Bis, and G6 6976 completely or almost completely inhibited PMA-induced PLD2-PKCα interaction, whereas rottlerin showed no inhibition at all. The large inhibitory effect of G6 6976 on the interaction seemed inconsistent with its minimal inhibition of PLD2 activity. We hypothesized that the relationship between the interaction between PKCα and PLD2 and the activation of PLD2 was not linear. In other words, only a small amount of PKCα binding was required for the full activation of PLD2. To prove this hypothesis, we expressed increasing amounts of PKCα together with PLD2 and examined the effects on the PLD2-PKCα interaction and PLD2 activity. As shown in Fig. 7F, the addition of increasing of PKCα cDNA from 0.08 to 0.48 μg led to a proportional increase in the PLD2-PKCα association. However, 0.48 μg of PKCα CDNA produced no further increase in PLD2 activity than did 0.08 μg. In other words, the relationship between PLD2-PKCα association and PLD2 activation was nonlinear.

Phosphorylation Suppresses PLD2 Activity—The above data indicated that protein-protein interaction is more likely than phosphorylation to be responsible for PLD2 activation by PMA/PKCα. The question then is, What is the role of phosphorylation in the control of PLD2 activity? Fig. 8A shows the changes of PLD2 activity after PMA treatment of COS-7 cells in the absence or presence of overexpressed PKCα. Without PKCα expression, the PLD product PtdBut accumulated linearly over 10 min in the presence of PMA, showing that PLD2 remained active during this period. However, in the presence of overexpressed PKCα, there was an increase in PtdBut before PMA treatment (0 min) followed by a slow increase with PMA. The latter change indicates that PLD2 is deactivated after several minutes of PMA treatment (0 min) followed by a slow increase with PMA. The former change indicates that PLD2 is activated after several minutes of PMA treatment (0 min) followed by a slow increase with PMA. This phosphorylation pattern is highly correlated with the deactivation of PLD2 activity. The in vitro assay of PLD2 in Fig. 8B shows that PLD2 was phosphorylated much earlier and stronger in the presence of overexpressed PKCα than in its absence. This phosphorylation pattern is highly correlated with the deactivation of PLD2 activity. The in vitro assay of PLD2 in Fig. 7A shows that the inclusion of ATP in the reaction with PKCα and PMA inhibited PLD2 activity and that the inhibition was abolished by the PKC inhibitor Ro-31-8220. These data indicate that phosphorylation can contribute to the suppression of PLD2 activity.

DISCUSSION

Previously our group showed that PLD2, like PLD1, exhibited a large response to PMA when overexpressed in COS-7 cells (21). This paper represents a follow-up of this observation. The present results show that PMA stimulates PLD2 activity up to 6-fold when overexpressed in COS-7 cells (Fig. 1), in contrast to a previous report (11) but in agreement with studies in HEK293 and Sf9 cells, where PLD2 also showed large responses to PMA or PKCα when overexpressed (19, 30).

A major issue in exploring the mechanism by which PMA/PKC activates PLD1 or PLD2 is the role of phosphorylation. It has been reported that PLD2 is Ser/Thr phosphorylated by PKCα after PMA treatment of rat pheochromocytoma PC12 cells (22). In this case the Ser/Thr phosphorylation was revealed by phosphoamino acid analysis. In another paper, the Ser/Thr phosphorylation of PLD2 was implicated by using oka-daic acid, an inhibitor of Ser/Thr protein phosphatases 1 and 2A (27). In the present study, using phospho-specific antibodies, we clearly demonstrate that PLD2 undergoes phosphorylation on both serine and threonine residues upon PMA treatment of COS-7 cells (Fig. 3A). Furthermore, we provide evidence that PKCα mediates the effect of PMA. This conclusion is based on the following points. First, G6 6976, a PKC inhibitor that is relatively specific for PKCα and PKCβ1, abol-
FIG. 7. PMA induces PLD2 binding with PKCα.

A, COS-7 cells were treated with 100 nM PMA for the indicated times, and subcellular fractions were prepared as described under “Experimental Procedures.” PKCα levels in the membrane (M) and cytosol (C) fractions were determined using Western blotting.

B, COS-7 cells were transfected with PKCα, Xpress vector, and/or Xpress-tagged PLD2 as indicated and treated with or without 100 nM PMA for 30 min. IP, immunoprecipitation; IB, Western blotting. C, COS-7 cells were transfected with Xpress-tagged PLD2 and PKCα and then treated with 100 nM PMA for the indicated times. D, COS-7 cells were transfected with Xpress-tagged PLD2 and then treated with 100 nM PMA for the indicated times. E, COS-7 cells were transfected with Xpress-tagged PLD2 and PKCα and then treated with or without 100 nM PMA in the presence or absence of 5 μM Ro-31-8220, 5 μM Bis, 0.5 μM Go6976, or 15 μM rottlerin as indicated. F, COS-7 cells were transfected with increasing amounts of PKCα together with Xpress-PLD2 and then treated with PMA as indicated. PLD activity, immunoprecipitation, and Western blotting were conducted as described under “Experimental Procedures.” Data are representative of at least three independent experiments. p-, phospho-.
Regulation of Phospholipase D by Protein Kinase C

The issue of whether or not phosphorylation is required for PLD activation still remains controversial. This issue has mostly involved studies of PLD1. A recent paper from our lab provides evidence that phosphorylation is not required for PLD1 activation (26), but there are reports to the contrary (23, 24). The requirement of phosphorylation for PKCα-mediated PLD2 activation by PMA was therefore examined in this study. Although our findings indicate that during PMA-induced activation PLD2 becomes heavily phosphorylated on both Ser and Thr residues (Fig. 3A), there is much evidence that this phosphorylation is not required for PLD2 activation. First, although Gia6 6976 eliminated the ability of PMA to phosphorylate PLD2 (Fig. 3B), it only exerted a slight inhibition on the activation of the enzyme (Fig. 2). Second, KN-PKCα, a PKCα kinase-dead mutant, still activated PLD2 robustly (Fig. 6A). Third, comparison between the time courses of the effects of PMA on PLD2 phosphorylation (Fig. 3A) and activation (Fig. 1A) showed that the activation of PLD2 by PMA occurred earlier than did phosphorylation. Fourth, by comparing the concentration curve of PMA-induced PLD2 activation (Fig. 5B) and the dose-response pattern of PMA-induced PLD2 phosphorylation (Fig. 5A) we found that PLD2 was activated by PMA at concentrations (0.5 and 1 nM) where phosphorylation of PLD2 didn’t occur yet. Based on the above findings, we have concluded that phosphorylation is not required for the activation of PLD2 by PMA.

Furthermore, our data suggest that the phosphorylation of PLD2 actually suppresses its enzyme activity. First, overexpression of the kinase-dead PKCα mutant showed a bigger stimulation of PLD2 activity than did overexpression of wild-type PKCα at the same level (Fig. 6A), implying that phosphorylation decreases PLD2 activity. Second, after the initial activation by PMA treatment of COS-7 cells, PLD2 lost activity faster in the presence of co-expression of PKCα than in its absence (Fig. 8A), and this is highly correlated with the fact that PLD2 undergoes phosphorylation much faster in the former case than in the latter (Fig. 8B). Third, in vitro PLD assay also showed that when ATP was included in the assay system, PLD2 activity decreased, and this decrease was abolished by the PKC inhibitor Ro-31-8220 (Fig. 8C). Consistent with our results, Watanabe and Kanaho (27) also demonstrate that phosphorylation leads to the inhibition of PLD2 activity.

PLD2 has been reported to bind to a lot of intracellular proteins. These proteins include the opioid receptor (31), phospholipase y1 (32), c-Src (33), epidermal growth factor receptor (34), and PKCδ (22). The binding is either ligand-dependent or -independent. PLD2 has also been reported to associate with PKCα in HEK-293 cells when both proteins were overexpressed, but this association was ligand-independent (30). Siddiqi et al. (19) report that when PLD2 and PKCα were both overexpressed in Sf9 cells, an association between them was observed and was increased by PMA. It is, therefore, possible that the protein-protein interaction between PLD2 and PKCα contributes to the activation of PLD2. In the present study, we showed that in COS-7 cells, an association between PLD2 and PKCα is also present, and this association was greatly enhanced by PMA (Fig. 7B). In addition, in the absence of PMA, KN-PKCa showed a stronger association with PLD2 than wild type PKCα (Fig. 6B). This is correlated with the fact that KN-PKCa induced a larger stimulation of PLD2 activity than did wild type PKCα (Fig. 6A). Furthermore, the effects of PKC inhibitors on PMA-induced PLD2 activation were correlated with their effects on PMA-induced PLD2-PKCα interaction. These observations support the conclusion that the PLD2-PKCα interaction is responsible for PLD2 activation. In contrast to PKCα, interaction of PKCδ and PKCe, the other two

**Fig. 8. Phosphorylation of PLD2 contributes to the suppression of its activity.** A. COS-7 cells were transfected with PLD2 together with PKCα or vector control. Cells were then treated with 100 nM PMA as indicated. The PLD2 activities shown are those after subtraction of endogenous PLD activity. B. COS-7 cells were transfected with PLD2 together with PKCα or vector control. Cells were then treated with 100 nM PMA for the times indicated. PLD2 phosphorylation was determined as described under “Experimental Procedures.” IP, immunoprecipitation; IB, Western blotting. C. membranes from PLD2-transfected COS-7 cells were obtained for in vitro PLD assay. ATP, PKCα, FMA, and Ro-31-8220 were included in the assay as indicated. In vitro PLD assays were conducted as described under “Experimental Procedures.” Data are representative of at least three independent experiments. p-, phospho-.
PMA-responsive PKC isoforms, with PLD2 was not detected (Fig. 7D). Also, overexpression of PKCβ or PKCε caused no stimulation of PLD2 activity (Fig. 6A). These data demonstrate that PMA induces a specific interaction between PKCα and PLD2 and that PKCα subsequently activates PLD2 by protein-protein interaction.

In conclusion, our findings show that PLD2 becomes Ser/Thr-phosphorylated during its activation by PMA and that PKCα mediates the effect. However, the phosphorylation of PLD2 is not required for its activation. Rather, the phosphorylation is related to a slow deactivation of PLD2. We suggest that a protein-protein interaction between PLD2 and PKCα is responsible for PLD2 activation by PMA in COS-7 cells.

Acknowledgments—We thank Judy Nixon for help in the preparation of this manuscript. We also thank Dr. Stanley Hoffman of Medical University of South Carolina for kindly providing the PKCε cDNA.

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