Protein Kinase Cα Translocates to the Perinuclear Region to Activate Phospholipase D1*

Received for publication, March 2, 2004, and in revised form, June 7, 2004
Published, JBC Papers in Press, June 8, 2004, DOI 10.1074/jbc.M402372200

Tianhui Hu and John H. Exton‡
From the Howard Hughes Medical Institute and Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37212

Phospholipase D (PLD)1 is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid and choline and is involved in many important intracellular processes such as vesicle trafficking in Golgi, exocytosis, endocytosis, cytoskeletal reorganization, respiratory burst, and protein expression (1). Two isoforms of mammalian PLD have been cloned. PLD1 can be regulated by many factors such as protein kinase C (PKC) and members of the Rho and Arf families of small G proteins (2–5), whereas PLD2 exhibits a high basal activity and shows little or no response to PKC, Rho, or Arf in vitro (6–8). PKCα is considered a major regulator of PLD1, and its role has been explored extensively. The PKC phosphorylation and interaction sites on PLD1 have been widely studied (9, 10). There are also several reports indicating that the PLD interaction sites on PKCα have may exist in both the N terminus (11, 12) and the C terminus (13). Previous studies have shown that phosphorylation is not required for the in vitro activation of PLD by PKCα (3, 13–15). However, the role of phosphorylation in the regulation of PLD in vivo remains uncertain.

Our recent work has provided in vivo evidence that PKCα activates PLD1 through a protein-protein interaction and that phosphorylation of PLD1 results in inactivation (13). However, other groups have provided evidence that phosphorylation of PLD1 is needed for its activation by PKCα. The effect of PKC inhibitors such as Ro-31-8220 on PLD1 activation has been considered one of the major pieces of evidence for a role of phosphorylation (for references, see Ref. 1). Ro-31-8220 (RO), an ATP analog and a potent PKC kinase inhibitor (16, 17), markedly inhibits PLD1 activation induced by 4β-phorbol 12-myristate 13-acetate (PMA) in vivo, consistent with the view that PKCα activates PLD1 by phosphorylation. However, the mechanism by which RO inhibits PLD1 activity has not been studied. As a potent inhibitor of PKCα, RO could also change PKCα autophosphorylation and affect its cellular localization. It could also block the interaction of PKCα with PLD1.

PLD1 exhibits variable patterns of subcellular membrane localization depending on the cell type (18). In mammalian cells, PLD1 is enriched in the perinuclear region, which may include the Golgi apparatus (18, 19). Some reports indicate that PLD1 also localizes to secretory granules, late endosomes, and lysosomes (18, 20–22). Cell fractionation studies show that PLD1 activity is restricted to caveolin-enriched membranes in some cell lines (23–25). There is other evidence that PLD1 might localize at the plasma membrane (26, 27). It is usually considered that after short term stimulation, PLD1 stays at its perinuclear location and does not undergo translocation. However, there is a report showing that upon longer time stimulation with antigen, PLD1 can translocate to the plasma membrane in RBL-2H3 cells (27). A recent report has also shown that PLD1 translocates from perinuclear endosomes and Golgi to the plasma membrane 2 h after PMA stimulation of COS-7 cells (20).

It has been established that PKCα is predominantly cytosolic but translocates to the membrane fraction after PMA stimulation (28). However, the exact subcellular location of PKC after stimulation varies depending on the cell line and stimulus. Some studies indicate that PKCα translocates from cytosol to plasma membrane after stimulation (29, 30) and concentrates in cell-cell contact areas (31), whereas other groups provide evidence that PKCα translocates to the nucleus after PMA stimulation (32). Other reports indicate that PKCα may translocate to both the plasma membrane and perinuclear structures that may be the endoplasmic reticulum (33, 34) or recycling endosomes (35). There is evidence that PKCα translocation is closely correlated with its phosphorylation status. For example, it has been shown that dephosphorylated PKCα translocates to the plasma membrane (35, 36) and vice versa (37).

The inhibition of phorbol ester activation of phospholipase D1 (PLD1) by protein kinase C (PKC) inhibitors has been considered proof of phosphorylation-dependent activation of PLD1 by PKCα. We studied the effect of the PKC inhibitors Ro-31-8220 and bisindolylmaleimide I on PLD1 activation and found that they inhibited the activation by interfering with PKCα binding to PLD1. Further studies showed that only unphosphorylated PKCα could bind to and activate PLD1 and that both inhibitors induced phosphorylation of PKCα. The phosphorylation status of either PLD1 or PKCα per se did not affect PLD1 activation in vitro. Immunofluorescence studies showed that PLD1 remained in the perinuclear region after phorbol ester treatment, whereas PKCα translocated from cytosol to both plasma membrane and perinuclear regions. Both Ro-31-8220 and bisindolylmaleimide I blocked the translocation of PKCα to the perinuclear region but not to the plasma membrane. Studies with okadaic acid suggested that phosphorylation regulated the relocation of PKCα from the plasma membrane to the perinuclear region. It is proposed that localization and interaction of PKCα with PLD1 in the perinuclear region is required for PLD1 activation and that PKC inhibitors inhibit this through phosphorylation of PKCα, which blocks its translocation.

* This work was partly supported by the Vanderbilt Ingram Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Rm. 831 Light Hall, Vanderbilt University School of Medicine, Nashville, Tennessee 37212. Tel.: 615-322-6494; Fax: 615-322-4381; E-mail: john.exton@vanderbilt.edu.
1 The abbreviations used are: PLD, phospholipase D; PKC, protein kinase C; RO, Ro-31-8220; Bis-I, bisindolylmaleimide I; PMA, 4β-phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2A; PtdBut, phosphatidylbutanol; OA, okadaic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein.
PKCa relocates from the plasma membrane to the perinuclear region (36). Another report indicates that a site-specific phosphorylation of PKCa induces resistance to translocation and down-regulation (37).

In this study, the effect of RO on PLD1 activity was studied and the results showed that RO inhibits PLD1 activation by interfering with the association of PKC with PLD. In addition, cell imaging provides evidence that PKCa and PLD1 colocalize in response to PMA.

**Experimental Procedures**

**Materials**—PMA, bovine serum albumin, Nonidet P-40, phosphatidylinositol 4,5-bisphosphate, protein phosphatase 2A (PP2A), microcystin, 2% gelatin solution, and horseradish peroxidase-conjugated secondary antibody were from Sigma. Dipalmitoylphosphatidylcholine, phosphatidylethanolamine, and phosphatidylbutanol (PtdBut) standard were from Avanti Polar Lipids Corp. c-α-DipalmitoylLα-1-Palmitoyl-9,10-ΔH(N)-Neildiphosphatidylcholine and 1Hmyristic acid were from PerkinElmer Life Sciences. Protein G-agarose beads, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, fetal bovine serum, Tris-glycine SDS-polyacrylamide gels, PCDNA3.1(+), pCDNA3.1(His A,B,C) vectors, and anti-Xpress monoclonal antibody were from Invitrogen. The transfection reagent FuGENE 6 and the protease inhibitor mixture were from Roche Applied Science. COS-7 cells were from American Type Culture Collection. Anti-PKCα monoclonal antibody was from BD Transduction Laboratories. Anti-phosphothreonine polyclonal antibody was from Zymed Laboratories Inc.. Plasmid and PCR product purification kits were from Qiagen. Anti-rhodamine red anti-mouse IgG and the SlowFade light antifade kit were from Molecular Probes. The transfection reagent FuGENE 6 and the protease inhibitor mixture were from Roche Applied Science. COS-7 cells were from American Type Culture Collection.

**In Vitro PLD Assay**—After 5 h of transfection, the cells that were present in the medium were serum-starved and transfected with FuGENE 6 according to the manufacturer’s instructions. The transfection reagent FuGENE 6 and the protease inhibitor mixture were from Roche Applied Science. COS-7 cells were from American Type Culture Collection.

**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 5% CO2. Six-well plates were seeded with 2 × 104 cells/well, and 10-cm dishes were seeded with 8 × 105 cells 24 h before transfection with FuGENE 6 according to the manufacturer’s instructions. The transfection reagent FuGENE 6 and the protease inhibitor mixture were from Roche Applied Science. COS-7 cells were from American Type Culture Collection.

**Subcellular Fractionation**—After transfection and starvation overnight, 10-cm dishes of COS-7 cells were washed once with ice-cold phosphate-buffered saline (PBS) and then harvested using lysis buffer (25 mM Heps, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor mixture). After 10 s of sonication two times, the cell lysate was first centrifuged at 500 g for 20 min before 50 min of PMA (100 nM) treatment and measuring the formation of [3H]PtdBut as a percentage of total labeled lipids as described before (38).

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE and Western blotting using anti-Xpress antibody and Western blotting using anti-PKCα antibody and then analyzed by 8% SDS-PAGE and Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under "Experimental Procedures." The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.

**Immunoprecipitation and Western Blotting**—COS-7 cells cultured in 10-cm plates were transfected and starved overnight as described above. The cells were washed once with ice-cold PBS and harvested using immunoprecipitation buffer containing 25 mM Heps, pH 7.2, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM KCl, 10 mM NaF, 10 mM Na3VO4, 1.2 mM Na2HPO4, 1% Nonidet P-40, and protease mixture. The cell suspension was sonicated for 10 s and then spun at 120,000 g for 45 min at 4 °C to separate the cytosolic and crude membrane fractions.

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE, followed by Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE, followed by Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE, followed by Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE, followed by Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.

**In Vitro PLD Assay**—For in vitro assay, the cells were lysed and immunoprecipitated using anti-PKCα antibody and then analyzed by 6% SDS-PAGE, followed by Western blotting using anti-PKCα antibody (upper panel) or anti-Thr(P) polyclonal antibody (lower panel). D, COS-7 cells were treated with PMA (100 nM), RO (1 μM), or Bis-I (1 μM) for 15 min before being lysed and fractionated into cytosol and membrane fractions as described under “Experimental Procedures.” The fractions were analyzed by 8% SDS-PAGE and Western blotting with anti-PKCα antibodies. Ct, control; C, cytosol; M, membrane. The data are representative of at least four experiments.
analysed by SDS-PAGE on 8% gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The blots were then blocked with 1% BSA and incubated with primary antibody followed by secondary antibodies. A lysate from cells overexpressing PKCa was used as a standard (right panel). C, COS-7 cells were treated with Bis-I (1 μM) for 30 min. The cells were lysed and immunoprecipitated using anti-Xpress antibody and Western blotted using both anti-Xpress and anti-PKCα antibodies. The bands were detected using ECL.

### RESULTS

The PKC Inhibitor Ro-31-8220 Inhibits PMA-stimulated PLD1 Activity by Interference with PLD1 and PKCa Association—The effects of different concentrations of RO on the time course of PtdBut accumulation in COS-7 cells stimulated with PMA are shown in Fig. 1A. The results indicate that RO almost completely inhibits PMA-stimulated PLD1 activity at a concentration of 1 μM. Because binding is essential for PKCa to activate PLD1 (13), we tested the effects of RO on PKCa-stimulated PLD1 and PKCa binding using coimmunoprecipitation. Fig. 1B shows that PMA increased the binding between PLD1 and PKCa and that RO blocked this binding at the concentration of 1 μM. Cell fractionation showed that only the membrane-associated fraction of PKCa bound to PLD1 (data not shown). The results indicate that RO can inhibit PKCa-stimu-
lated PLD1 activity by interference with PLD1 and PKCa binding.

RO Blocks PMA-stimulated PLD1 and PKCa Binding by Phosphorylation of PKCa—The finding that RO blocked PLD1 and PKCa binding was unexpected. As an ATP analog, RO may also have effects on PKCa autophosphorylation. PKCa exhibits different phosphorylation states with varying effects on activity (40). We therefore determined the influence of the phosphorylation status of PKCa on its binding to PLD1. The experiment employed a gel system that resolved phosphorylated PKCa from the nonphosphorylated form. The results show that, compared with the PKCa standard that showed both phosphorylated and nonphosphorylated forms, as verified by blotting with anti-ThrP) antibodies (not shown), only the less phosphorylated, fast moving band of PKCa bound to PLD1 (Fig. 1B). The effect of RO on PKCa autophosphorylation was also tested, and the results are shown in Fig. 1C. Both the band shift and the Thr phosphorylation results show that RO induced autophosphorylation of PKCa. The data also show that RO did not modify the phosphorylation of PKCa induced by PMA. Fig. 1D shows that RO induced the membrane translocation of PKCa.

To gain support for the view that the effect of RO on PLD1 activity and PLD1 and PKCa binding is due to its effect on PKCa and not to some nonspecific effect, Bis-I, another PKCa kinase inhibitor with a similar structure to RO (17) was tested. Fig. 2A shows that Bis-I completely inhibited PMA-stimulated PLD1 activity at a concentration of 1 μM and also blocked the PMA-stimulated PLD1 and PKCa binding (Fig. 2B). In agreement with the results of Fig. 1B, only the less phosphorylated form of PKCa bound to PLD1 (Fig. 2B). Like RO, Bis-I increased the autophosphorylation of PKCa and caused minimal inhibition of the effect of PMA (Fig. 2C). Bis-I also induced the membrane translocation of PKCa (Fig. 1D).

The Phosphorylation Status of Either PLD1 or PKCa Does Not per Se Affect PMA-stimulated PLD1 Activity in Vitro—The preceding results showed that phosphorylated PKCa was unable to bind to PLD1 and that RO and Bis-I inhibited PLD1 and PKCa binding by phosphorylating PKCa and making it unable to bind to PLD1. To see whether the phosphorylation status of either PLD1 or PKCa would affect in vitro PLD1 activity, the Ser/Thr phosphatase PP2A was used to treat membranes containing PLD1 and cytosol containing PKCa for 30 min before conducting an in vitro PLD assay. Pretreatment of PP2A with microcystin, a potent PP2A inhibitor (39) was used as a control. The results shown in Fig. 3A clearly indicate that whether or not PLD1, PKCa, or both were treated with PP2A, the PLD1 activity remained the same when assayed in vitro. To check whether PP2A and microcystin functioned as expected, PKCa

![Colocalization of PLD1 and PKCa in COS-7 cells](image1)

**Fig. 4. Colocalization of PLD1 and PKCa in COS-7 cells.** COS-7 cells were cotransfected with Xpress PLD1 and GFP-PKCa and starved 5 h later. After 24 h of starvation, the cells were treated with PMA (300 nM) for 15 min and stained as described under “Experimental Procedures.” The data are representative of at least five separate experiments. Bar, 15 μm.

![The effect of RO and Bis-I on PMA stimulated PKCa translocation](image2)

**Fig. 5. The effect of RO and Bis-I on PMA stimulated PKCa translocation.** COS-7 cells were transfected with GFP-PKCa and starved 5 h later. After 24 h of starvation, the cells were pretreated with RO (500 nM) or Bis-I (500 nM) for 30 min and then treated with PMA (300 nM) for 15 min. After washing and fixation, the cells were stained as described under “Experimental Procedures.” The data are representative of at least five separate experiments. Bar, 15 μm.
A previous report has indicated that PKCa perinuclear translocation may be due to its dephosphorylation following its translocation from the cytosol to the plasma membrane (36). To explore the possible role of dephosphorylation, okadaic acid (OA), a PP2A inhibitor was used to treat the cells, and its effects on PLD1 activity and PKCa translocation were observed. Fig. 6A shows that OA partially inhibited 5 nM PMA-stimulated PLD1 activity in a concentration-dependent manner. Concentrations of OA higher than 500 nM were not tested because the cells showed loss of viability. The effects of OA (500 nM) on PMA-stimulated PKCa phosphorylation were also tested, and Fig. 6B shows the expected increase. Fig. 6C also shows that OA partially blocked PKCa translocation to the perinuclear region. These results support the view that the translocation of PKCa from cytosol to perinuclear region involves its dephosphorylation.

**DISCUSSION**

The present study arose from an effort to find if the inhibitory effect of RO on PLD1 activity involved an inhibition of PKCa phosphorylation of PKCa on its interaction with PLD1 observed in vivo is not due to the phosphorylation per se but is dependent on other cellular components.

Effects of Okadaic Acid on PMA Activation of PLD1 and Translocation of PKCa to the Perinuclear Region—A previous report has indicated that PKCa perinuclear translocation may be due to its dephosphorylation following its translocation from the cytosol to the plasma membrane (36). To explore the possible role of dephosphorylation, okadaic acid (OA), a PP2A inhibitor was used to treat the cells, and its effects on PLD1 activity and PKCa translocation were observed. Fig. 6A shows that OA partially inhibited 5 nM PMA-stimulated PLD1 activity in a concentration-dependent manner. Concentrations of OA higher than 500 nM were not tested because the cells showed loss of viability. The effects of OA (500 nM) on PMA-stimulated PKCa phosphorylation were also tested, and Fig. 6B shows the expected increase. Fig. 6C also shows that OA partially blocked PKCa translocation to the perinuclear region. These results support the view that the translocation of PKCa from cytosol to perinuclear region involves its dephosphorylation.

**DISCUSSION**

The present study arose from an effort to find if the inhibitory effect of RO on PLD1 activity involved an inhibition of PKCa phosphorylation of PKCa on its interaction with PLD1 observed in vivo is not due to the phosphorylation per se but is dependent on other cellular components.

Effects of Okadaic Acid on PMA Activation of PLD1 and Translocation of PKCa to the Perinuclear Region—A previous report has indicated that PKCa perinuclear translocation may be due to its dephosphorylation following its translocation from the cytosol to the plasma membrane (36). To explore the possible role of dephosphorylation, okadaic acid (OA), a PP2A inhibitor was used to treat the cells, and its effects on PLD1 activity and PKCa translocation were observed. Fig. 6A shows that OA partially inhibited 5 nM PMA-stimulated PLD1 activity in a concentration-dependent manner. Concentrations of OA higher than 500 nM were not tested because the cells showed loss of viability. The effects of OA (500 nM) on PMA-stimulated PKCa phosphorylation were also tested, and Fig. 6B shows the expected increase. Fig. 6C also shows that OA partially blocked PKCa translocation to the perinuclear region. These results support the view that the translocation of PKCa from cytosol to perinuclear region involves its dephosphorylation.

**DISCUSSION**

The present study arose from an effort to find if the inhibitory effect of RO on PLD1 activity involved an inhibition of PKCa phosphorylation of PKCa on its interaction with PLD1 observed in vivo is not due to the phosphorylation per se but is dependent on other cellular components.
phosphorylation or not. Our results show that RO inhibits PLD1 activity by blocking the PMA-stimulated association of PKCα with PLD1 (Fig. 1). Previous studies have shown that PKCα auto-phosphorylation is closely related to its activity and that the enzyme exists in different phosphorylation states (36, 37, 40, 41). Our study found that only the unphosphorylated form of PKCα can bind to PLD1. This finding provides a clue as to why RO inhibits the binding of PKCα to PLD1. Fig. 1D shows that treatment with RO causes phosphorylation of PKCα, and this is associated with its inability to bind to PLD1. Similar results were obtained with Bis-I, another kinase inhibitor of PKCα (Fig. 2). These results support the view that PKCα can activate PLD1 by a protein-protein interaction but that only unphosphorylated PKCα can bind to PLD1.

A surprising result was the stimulation of the phosphorylation of PKCα exerted by RO (Fig. 1C) and Bis-I (Fig. 2C) in vivo. Because it is very unlikely that these inhibitors, which are ATP analogs and act by competing with ATP, would directly stimulate the phosphorylation of PKCα, it seems that another protein kinase could be involved, as discussed below. Alternatively, the inhibitors may act by blocking the dephosphorylation of PKCα, which has been observed in some cell lines (36, 41). Another surprising result was that RO and Bis-I induced the translocation of PKCα to the membrane fraction (Fig. 1D). However, because of the opposite effects of the inhibitors and PMA on the activation of PLD1, it seems unlikely they both translocated PKCα to the same membrane(s). In fact, Fig. 5 shows that the inhibitors did not induce the same intracellular translocations of PKCα as those seen with PMA.

A key issue to be resolved in the present study is why PMA, which induced phosphorylation of PKCα, caused activation of PLD1 and promoted the association of PKCα with PLD1 i.e. changes opposite to those observed when PKCα phosphorylation was increased by RO or Bis-I (Figs. 1 and 2). One obvious explanation is that the protein kinase (or protein phosphatase) involved in the phosphorylation induced by the inhibitors differs from PKCα. This could result in the phosphorylation of different residues. As described above, the intracellular localization of PKCα seen with PMA differed from that seen with the inhibitors. Although there could be many reasons for this, it could reflect a difference in PKCα phosphorylation (36, 37, 41, 44). However, we cannot provide definitive proof of this hypothesis because the kinase (phosphatase) involved in the inhibitor effects has not been identified.

The translocation experiments provide an explanation of how PMA might induce the activation of PLD1 by a protein-protein interaction. They confirm many observations that PLD1 is located predominantly, but not exclusively, in the perinuclear region (7, 19, 27, 42, 43) but, more importantly, illustrate that PMA causes the translocation of PKCα to the perinuclear region as well as to the plasma membrane (Fig. 4 and Ref. 35) and that RO and Bis-I block the localization of PKCα to the perinuclear region (Fig. 5). Because our present and previous data (13) indicate that the association of PKCα with PLD is required for PMA activation of PLD1, their colocalization in the perinuclear region would be expected to lead to PLD1 activation. Likewise, the inhibition of this colocalization by RO and Bis-I could explain why these inhibitors block the activation.

The results with RO and Bis-I support previous findings that the phosphorylation status of PKCα is closely related with its cellular localization (36, 37, 41, 44), although some of these studies did not define the membrane fraction(s) involved. Our results also show that RO and Bis-I phosphorylate PKCα, and it is hypothesized that this makes it unable to translocate to perinuclear region. Phorbol esters presumably activate PKCα at the plasma membrane. Thus it is likely that PKCα initially translocates to the plasma membrane and subsequently relocates to the perinuclear region (36). There is evidence that this relocation is due to its dephosphorylation by PP2A and can be blocked by RO (36). This provides support for the idea that RO and Bis-I block the PKCα relocation to the perinuclear region by increasing its phosphorylation, thus inhibiting its binding to and activation of PLD1. The inhibitors would be expected to block the phosphorylation of PKCα induced by PMA. However, it is evident from Figs. 1C and 2C that they induce phosphorylation of PKCα per se and have little or no effect on the phosphorylation induced by PMA. As discussed above, it is likely that the inhibitors act through another protein kinase or a protein phosphatase.

To further prove the relationship between PKCα phosphorylation and its localization, OA, a PP2A inhibitor, was used to study its effect on PKCα translocation and activation of PLD1. The results of Fig. 6 showed that OA partially inhibited PKCα relocation to the perinuclear region and thus inhibited the activation of PLD1. The results with RO, Bis-I, and OA suggest PMA induced PKCα translocation to the plasma membrane and then relocation to the perinuclear region to activate PLD1.

In our study PKCα was tagged with GFP to track its translocation in COS-7 cells. Although GFP has been widely used in many previous reports to study the localization and translocation of PKC, we considered the possible effect of GFP on PKCα localization. The GFP results showed that when GFP alone was expressed it localized inside nuclei (data not shown). When the translocation of GFP-PKCα was analyzed using cell fractionation, the results showed that GFP-PKCα had the same translocation ability as nontagged PKCα (data not shown). We also compared the PLD activity increase induced by nontagged PKCα or GFP-tagged PKCα and found no difference (data not shown). Therefore we conclude that GFP tagging does not affect the ability of PKCα to translocate or activate PLD.

In summary, the present findings present a novel mechanism by which PKCα could activate PLD1 in vivo. They also illustrate that only the nonphosphorylated form of PKCα can interact with PLD1 and that PKCα and PLD1 can colocalize in the perinuclear region following PMA stimulation. They also reveal some unexpected findings with respect to two widely used PKC inhibitors, namely that they induce phosphorylation of PKCα in vivo and block its interaction with PLD1. We propose this as a mechanism by which they inhibit PMA activation of PLD1.

Acknowledgment—We thank Judy Nixon for preparation of the manuscript.

REFERENCES
1. Exton, J. H. (2002) Rev. Physiol. Biochem. Pharmacol. 144, 1–94
2. Hammond, S. M., Altschuller, Y. M., Sung, T. C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29645
3. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G. D., Frohman, M. A., and Morris, A. J. (1997) J. Biol. Chem. 272, 3860–3868
4. Park, S. K., Provoost, J. J., Bae, C. D., Ho, W. T., and Exton, J. H. (1997) J. Biol. Chem. 272, 29263–29271
5. Colley, W. C., Altschuller, Y. M., Sue-Ling, C. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Branch, K. D., Turka, S. E., Bollag, R. J., Bollag, W. B., and Frohman, M. A. (1997) In Biochem. J. 326, 745–753
6. Kodaki, T., and Yamashita, S. (1997) J. Biol. Chem. 272, 11408–11413
7. Glay, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altschuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) Curr. Biol. 7, 191–201
8. Liao, H., Arnold, R. S., and Lambeth, J. D. (1998) J. Biol. Chem. 273, 12846–12852
9. Kim, Y., Han, J. M., Park, J. B., Lee, S. D., Oh, Y. S., Chung, C., Lee, T. G., Kim, J. H., Park, S. K., You, J. S., Suh, P. G., and Ryu, S. H. (1999) Biochemistry 38, 10344–10351
10. Zhang, Y., Altschuller, Y. M., Hammond, S. M., Hayes, F., Morris, A. J., and Frohman, M. A. (1999) EMBO J. 18, 6329–6348
11. Singer, W. D., Brown, H. A., Jiang, X., and Sternweis, P. C. (1996) J. Biol. Chem. 271, 4504–4510
12. Sciorra, V. A., Hammond, S. M., and Morris, A. J. (2001) Biochemistry 40,
