Cloning and Characterization of Phospholipase D from Rat Brain*

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The regulation of phospholipase D cloned from rat brain (rPLD) was examined in vivo and in vitro. The enzyme was a shorter splice variant of human phospholipase D1 (Hammond, S. M., Altshuller, Y. M., Sung, T.-C., Rudge, S. M., Rose, K., Engebrecht, J. A., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29643). Its expression in COS-7 cells led to increased phospholipase D (PLD) activity that was further stimulated by constitutively active V14RhoA. V14RhoA had no effect on the endogenous PLD of the COS-7 cells, but constitutively active L71ARF3 increased its activity. In contrast, L71ARF3 did not activate rPLD expressed in the cells. Addition of phorbol ester markedly increased the endogenous PLD activity of COS-7 cells, and there was a further increase in the cells expressing rPLD.

In membranes from COS-7 cells expressing rPLD, addition of myristoylated ADP-ribosylation factor (ARF) and RhoA in vitro stimulated PLD activity. The effect of ARF was greater than that of RhoA, although the concentrations for half-maximal stimulation (0.08–0.2 μM) were similar. Membranes isolated from cells expressing rPLD plus L71ARF3 and/or V14RhoA also showed higher PLD activity but no synergism between the two G proteins. Addition of phorbol ester and protein kinase C α (PKCα) also stimulated PLD activity in membranes from COS-7 cells expressing rPLD, but it had no effect on the activity in control (vector) membranes and did not enhance the effects of constitutively active ARF or Rho. The stimulation by PKCα did not require ATP and was not increased by addition of this nucleotide. No synergism between ARF and Rho and between these and PKCα on PLD activity was observed when these were added to membranes from cells expressing rPLD. Oleate inhibited the PLD activity of membranes from both control and rPLD-expressing cells.

In summary, these results indicate that in vitro, rPLD is stimulated by ARF, RhoA, and PKCα and inhibited by oleate. However, in intact COS-7 cells, ARF activates endogenous PLD but not rPLD, whereas the reverse is true for RhoA. In addition, the effects of phorbol ester are much greater in the intact cells. It is concluded that the regulation of rPLD in intact COS-7 cells differs significantly from that seen in vitro; possible reasons for this are discussed.

Phospholipase D (PLD) is believed to play a role in signal transduction in many cell types because it is a ubiquitous enzyme that is regulated by a great variety of hormones, neurotransmitters, growth factors, cytokines, and other molecules involved in extracellular communication (Ref. 1 and references therein). The product of PLD, phosphatidic acid (PA) has been proposed to function in mitogenesis in fibroblasts, stimulation of respiratory burst in neutrophils, regulation of secretion, and activation of specific protein kinases and other proteins. Diacylglycerol, which can be formed from PA by phosphatidic acid phosphatase, is a major regulator of protein kinase C (PKC). Lysophosphatidic acid, which can be produced from PA through hydrolysis by a specific phospholipase, A₄, is now recognized as an important extracellular signal.

Studies employing PKC inhibitors or down-regulation of the enzyme indicate the involvement of PKC in agonist regulation of PLD in many cell types (1). The stimulation of PLD by PKC may occur by phosphorylation-dependent (2) or phosphorylation-independent (3–6) mechanisms. On the other hand, PKC-independent control of PLD by agonists has been observed in some studies (Ref. 1 and references therein).

Early studies indicated that the PLD activity of neutrophils or HL60 cells was enhanced by GTPγS in combination with a cytosolic protein, which was subsequently identified as ARF (7, 8). ARF was originally discovered as a factor required for the ARF-ribosylation of Gsα by cholera toxin and was later shown to be involved in protein trafficking in the Golgi apparatus (9). Stimulation of PLD activity by ARF has now been observed using recombinant PLD and partially purified preparations of the enzyme from various tissues (Ref. 1 and references therein). ARF translocation to the membrane fraction has also been correlated with PLD activation after treatment of HL60 cells with phorbol ester or formyl-methionyl-leucyl-phenylalanine (10). Similar findings have been reported by Rümenapp et al. (11) in HEK cells expressing muscarinic receptors and treated with carbachol.

Extraction of Rho from membrane fractions by RhodGDI treatment reduces PLD activity in response to GTPγS, and the response can be recovered by the addition of Rho proteins (12–15). Rho proteins are involved in control of the actin cytoskeleton and mediate changes in response to extracellular signals, such as lysophosphatidic acid and growth factors (16, 17). Recently, Rho-binding proteins have been identified (protein kinase N, rhophilin, rhotekin, and Rho kinase) (16, 17). Rho also activates phosphatidylinositol 4-phosphate 5-kinase (18), which synthesizes phosphatidylinositol 4,5-bisphosphate (PIP₂), a cofactor for PLD activation (7, 19).

Cytosolic proteins have been reported to stimulate PLD syn-

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‡ The abbreviations used are: PLD, phospholipase D; hPLD1, human PLD 1; rPLD, rat PLD; PA, phosphatidic acid; PKC, protein kinase C; GTPγS, guanosine 5′-O-(3-thiotriphosphate); ARF, ADP-ribosylation factor; PIP₂, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; PC, dipalmitoylphosphatidylcholine.
ergistically with ARF and/or Rho (5, 15, 20–24), and one of these is PKCα (5). Inhibitory proteins have also been identified (25–29). These include fodrin (28) and synaptotagmin, which acts indirectly by dephosphorylating PIP2 (25, 29). The in vivo significance of these stimulatory and inhibitory proteins has not been assessed.

There is much indirect evidence that PLD exists as several isozymes. This is based on differences in substrate specificity; in the regulation of the enzyme from different tissues and subcellular fractions by PKC, PIP2, fatty acids, Rho, and ARF; and in the effects of divalent cations, detergents, and pH (1, 30, 31). Although it is probable that the regulation of PLD is complex, the results strongly suggest the presence of several isozymes. Recently, human PLD (hPLD1) was cloned from HeLa cells and characterized in vitro (32, 33). To determine whether this enzyme is similar to that in other mammalian tissues, we cloned rPLD from a rat brain cDNA library and determined its regulation by ARF, RhoA, and PKC in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), sodium oleate, and PA were purchased from Sigma. Dipalmitoylphosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylethanol, and phosphatidylbutanol (PtdBut) were purchased from Avanti Polar Lipids Corp. GTP-β-S was obtained from Boehringer Mannheim. [32P]HPhosphatidylcholine and [9,10-3H]Myristic acid were purchased from NEN Life Science Products. PKCα was purchased from Panvera Corp. Phosphatidylinositol 4,5-bisphosphate (PIP2), RhoA, and recombinant myristoylated ARF3 (mARF) were prepared as described previously (31). SDF-polyacrylamide gels were purchased from Novex. [α-32P]dTTP, hybridization solution, and random DNA labeling kit were from Amersham. Monoclonal antibody against RhoA from mouse was from Santa Cruz, and the cDNA for RhoA was generously provided by Dr. R. Cerione (Cornell University, Ithaca, NY). Anti-sARFII, polyclonal antibody from rabbit (which recognizes ARF1 and ARF3), and the cDNA for ARF3 were kind gifts of Dr. J. Moss (National Institutes of Health, Bethesda, MD). A polyclonal antibody to the carboxy-terminal 12 residues of hPLD1 from rabbit was a kind gift of Dr. S. H. Ryu (Postech, Pohang, Korea). Poly(A)+ RNA from various rat tissues (CLONTECH) was hybridized with a probe excised from rPLD in pBluescript SK by NotI in the multiple cloning site of the vector and NsiI in rPLD. It was labeled with [32P]dTTP by random priming and contained 108 base pairs of the rPLD coding sequence, corresponding to amino acid residues 231–590. Hybridization was performed at 42 °C for 16 h in the presence of 40% formamide and 10% dextran sulfate. After hybridization, the membrane was washed at 50 °C in the presence of 0.1× SSC buffer and 1% SDS. Visualization was by exposure to either a phosphor storage screen (Molecular Dynamics) or Kodak Biomax MS film. After hybridization was performed on RNA from cell lines, total RNA was extracted using Trizol (Life Technologies, Inc.) according to the manufacturer’s instructions. Poly(A)+ RNA was purified by annealing with biotinylated oligo(dT)20 and SDF-harboring phages with magnetic beads. The RNA was separated by agarose gel electrophoresis and transferred to Nytran membranes (Schleicher & Schuell).

Measurement of PLD Activity in Membranes and Cytosol from Transfected COS Cells—Cells were transfected with either vector or rPLD as described earlier were washed twice with Buffer A (20 m Hepes (pH 7.2), 0.5 mM CaCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin) and scraped into 2.7 ml of Buffer A per 6-well tissue culture plate. The cells were homogenized by 20 passes in a Dounce tissue grinder. Unbroken cells were removed by centrifugation at 500 × g for 5 min. Crude membranes and cytosol were then isolated after centrifugation at 100,000 × g for 1 h. The resulting pellet (crude membranes) was resuspended and washed in Buffer A. PLD activity was measured by the formation of [3H]PtdBut from [3H]PtdSer phospholipid vesicles containing phosphatidylethanolamine/PtdSer/HPC (16:1:4:1) were used as substrate (7).

RESULTS

Cloning of Rat Brain PLD cDNA—Rat brain PLD cDNA was obtained from a rat brain cDNA library as described under “Experimental Procedures” using a DNA fragment of the
hPLD1 gene from HL60 cells. Using this approach, two full-length cDNA clones were obtained that had the translation initiation codon ATG, an in-frame stop codon, and a poly(A) tail. Both had identical sequences and were named rPLD. Analysis of the cDNA predicted an open reading frame encoding a 1036-amino acid protein with a calculated molecular mass of 119 kDa. The sequence showed 87% amino acid identity with hPLD1 (32) (Fig. 1) and had no recognizable motifs. The most striking difference from hPLD1 was that rPLD had a 38-amino acid deletion corresponding to residues 585–622 of hPLD1. To determine whether this region was species-specific or not, polymerase chain reaction was performed on the cDNA of HL60 cells using a primer spanning this area. Two kinds of polymerase chain reaction fragment were amplified, i.e., one with the insertion present and one with it absent (data not shown). These results indicate that the insertion is not species-specific and is probably a splicing variant, in agreement with a recent report (33).

Tissue Distribution of rPLD Transcript—Northern hybridization using mRNA from various rat tissues revealed the presence of a 5.3-kilobase transcript in all tissues examined (Fig. 2). It was highest in lung, brain, and kidney and weakly present in testis (Fig. 2). Kidney contained an additional band of 3.8 kilobases. The transcript was not detected in COS-7 cells but was evident in C6 glioma cells, Rat-1 fibroblasts, and PC12 pheochromocytoma cells (data not shown).

Stimulation of rPLD by Constitutively Active ARF and Rho in COS-7 Cells—There are many reports that PLD is regulated by the small GTP-binding proteins ARF and Rho (1). To test the in vivo regulation of rPLD by these proteins, rPLD was expressed in COS-7 cells. A recombinant protein of approximately 120 kDa was detected in the membranes by an antibody raised to a peptide corresponding to the carboxyl terminus of hPLD1 (Fig. 3) but not by a antibody raised to a sequence (residues 525–541) in hPLD1 that differs from that in rPLD (data not shown). Lesser amounts of the enzyme were also detected in the cytosol.2 The enzyme had a molecular mass of 120 kDa as determined by SDS-polyacrylamide gel electrophoresis (not shown), which corresponds to the value of 119 kDa deduced from the sequence. The antibody cross-reacted with a 98-kDa protein that was endogenously present in the COS-7 cells (Fig. 3). Although this protein could represent an endogenous PLD, it did not cross-react with antibodies raised to PLD2 or to other sequences in hPLD1 or rPLD (data not shown).

When the cells were transfected with wild-type forms of ARF3 and RhoA with or without rPLD, the pattern of PLD activity was low and was enhanced 2–3-fold by transfection with rPLD (Fig. 4 A). To study the regulation of rPLD in vivo, COS-7 cells were transfected with constitutively active forms of ARF and RhoA (L71ARF3 or V14RhoA) with and without rPLD. Western blotting showed increased expression of both of these small G proteins in the cytosol of the COS-7 cells (Fig. 3). V14RhoA was also detected in the membranes, but L71ARF3 was barely detectable in this fraction (Fig. 3). Cells transfected with only L71ARF3 showed a 2-fold increase of PLD activity, indicating that the endogenous PLD was responsive to ARF. In contrast, cells transfected with V14RhoA showed no significant increase of basal PLD activity. However, cells transfected with rPLD plus V14RhoA or rPLD plus L71ARF showed increases over basal PLD activity of 8.5- and 4.6-fold, respectively (Fig. 4 A).

There were no significant differences in the incorporation of [3H]myristic acid into phospholipids in the variously transfected cells, indicating that [3H]PtdBut production was a valid means of assaying PLD activity (data not shown).

When the cells were transfected with wild-type forms of ARF3 and RhoA with or without rPLD, the pattern of PLD

2 The amount of enzyme in the cytosol was variable. Compare Fig. 3 with Fig. 4 C.

Fig. 1. Comparison of human and rat brain PLD sequences.
The upper sequence (hPLD1) is of the enzyme cloned from HeLa cells (32). The lower sequence (rPLD) is that of the PLD cloned in the present study. Nonidentical residues are shown in black boxes.
activity (Fig. 4B) was similar to that seen with the constitutively active forms of the small G proteins (Fig. 4A). As discussed below, the stimulatory effects of the transfected wild-type G proteins can be attributed to factors present in the serum, because the stimulations were much less in serum-free medium (data not shown). When both ARF3 and RhoA were transfected together with rPLD, no synergistic interaction between the two G proteins was observed (Fig. 4B). In these experiments, rPLD expression in the co-transfections was similar to rPLD expression in the transfections with rPLD alone (Fig. 4C).3 The expression of the small G proteins was similar to that seen in Fig. 3 except that ARF was not detectable in the membranes.

Stimulation of rPLD activity by PMA in COS-7 Cells—The involvement of PKC in agonist stimulation of PLD activity differs among cell types (1). To see whether rPLD responds to the PKC activator PMA, COS-7 cells transfected with vector or rPLD were treated with 100 nM PMA. PMA produced a very large stimulation of the PLD activity of COS-7 cells, which was enhanced in the cells expressing rPLD (Fig. 5). The changes with PMA in both types of cell were substantial at 5 min and maximal at 30 min. These findings suggest that both the endogenous PLD of COS-7 cells and rPLD are activated by PKC in vivo.

Stimulation of rPLD in COS-7 Cell Membranes and Cytosol by ARF and RhoA in Vitro—To further investigate the regulation of rPLD by RhoA and ARF, COS-7 cells were transiently transfected with either vector alone or rPLD, and cell fractions were prepared and assayed for PLD activity as described under “Experimental Procedures.” In both membranes and cytosol, the basal PLD activity of rPLD-transfected cells was at least 2-fold higher than that of control cells (Fig. 6) and was increased by addition of GTPγS. When ARF was included with GTPγS in the assay, the PLD activity of both fractions was increased in both control and rPLD-transfected cells. However, the rPLD-transfected cells showed a much greater stimulation than the control cells (Fig. 6). In comparison to ARF, exogenous RhoA stimulated the PLD activity of membranes from rPLD-transfected cells but not that of control membranes (Fig. 6A). The stimulation by RhoA was consistently less than by ARF, and no stimulation by added RhoA was evident in cytosol from either control or rPLD-transfected cells (Fig. 6B).

Preloading RhoA with GTPγS prior to the PLD assay did not increase the magnitude of its effect (data not shown).

To determine the concentration dependence of activation of
rPLD by RhoA and ARF, transfected membranes were incubated with GTPγS and increasing concentrations of either RhoA or mARF. Both proteins stimulated rPLD in a dose-dependent manner, but ARF gave a much greater (8-fold) stimulation of rPLD than RhoA (2.5-fold) at maximal concentrations (compared with GTPγS alone). The half-maximally effective concentrations of RhoA and ARF were 80 and 200 nM, respectively.

**Stimulation of rPLD in COS-7 Cell Membranes by PKCa and Phorbol Ester in Vitro**—Membranes from both control and rPLD-transfected COS-7 cells were incubated with various combinations of PKCa, PMA, and ATP (Fig. 7). In the control (vector) cells, addition of PMA and/or PKCa had no effect. However, in the rPLD-transfected cells, PMA increased PLD activity 2-fold over basal. The PMA stimulation of rPLD was seen immediately after addition, was linear for 20 min, and continued to increase up to 60 min (data not shown). PLD stimulation by PMA alone indicates that these membranes contained a PKC isozyme(s). When purified PKCa was incubated with membranes from rPLD-transfected cells, a 3-fold increase in PLD was seen, and when both PMA and PKCa were added together, the PLD activity was no higher than with PKCa alone (Fig. 7), suggesting that the membranes contained activators of PKC. Surprisingly, the PKC effect was independent of ATP because measurement of this nucleotide in the incubations using a bioluminescence luciferase assay showed a concentration of 0.3 nM, which is far below the Kₘ of the enzyme for ATP (6 μM) (35). Furthermore, there was no further stimulation when ATP or its nonhydrolyzable analogs were added along with PKCa and PMA (Fig. 7 and data not shown).

Membranes from COS-7 cells that had been transfected with rPLD, L71ARF3, or V14RhoA were assayed for PLD activity using the in vitro assay system to see whether the results agreed with those observed in vivo. The membranes from the rPLD-transfected cells showed higher PLD activity than vector controls (Fig. 8A), although the magnitude of the increase was not as great as that seen in vivo (Fig. 4A). Co-transfection of L71ARF or V14RhoA resulted in small but reproducible further increases in activity, which were not synergistic (Fig. 9). The addition of GTPγS slightly enhanced the effects of the constitutively active small G proteins (data not shown). The addition of PKCa plus PMA produced a further stimulation of PLD activity in the rPLD-transfected cells (Fig. 8A), but no synergy with L71ARF or V14RhoA was observed. Western blotting showed that co-expression with the small G proteins did not alter rPLD levels in the cells (Fig. 8B).

To further test for synergism, membranes from COS-7 cells transfected with rPLD were incubated with GTPγS, ARF, RhoA, and PKCa alone or in various combinations (Fig. 9). Fig. 9 shows that the combination of ARF and RhoA with GTPγS produced an additive effect on PLD activity, but not synergism. Likewise, the combination of PKCa with either ARF or RhoA plus GTPγS did not result in a synergistic effect. In fact, inhibition was consistently observed when all three agents were combined (compare GTPγS plus RhoA, ARF, and PKCa with GTPγS plus RhoA and ARF).

**Oleate Inhibition of rPLD**—Sodium oleate is known to both activate and inhibit PLD activity depending on the tissue source of the enzyme (1, 30). Therefore, the effect of oleate on rPLD activity was investigated. As seen in Fig. 10, oleate inhibited both control and ARF-stimulated rPLD activity. Oleate inhibition of basal and ARF-stimulated PLD activity was also observed in control (vector) membranes, but the changes were of much lower magnitude.

**DISCUSSION**

The PLD cloned from rat brain (rPLD) is closely related to hPLD1 cloned from HeLa cells. It shows 87% amino acid sequence identity with the human enzyme; the major difference is a 38-amino acid deletion (residues 585-624). When this deletion is omitted, the sequence identity rises to 91%, and when homologous replacements are also included, the proteins are 95% similar. The deletion is not species-specific, because it was also detected in human placenta. Furthermore, a very recently published report (33) has shown the existence of a variant of hPLD1 that has the same deletion and shows 90% amino acid identity with rPLD. The short and long forms of the enzymes probably arise from the alternative splicing of exons, although efforts to demonstrate this by Southern analysis were inconclusive (data not shown).

Northern hybridization indicated a 5.3-kilobase transcript for rPLD that was present in all tissues examined, although it was only weakly detectable in liver and testis (Fig. 2). Kidney contained an additional 3.8-kilobase transcript, which probably corresponds to a closely related PLD. Western blotting of tissues with antibody to the carboxyl terminus of hPLD1 was not performed because, although the antibody recognizes rPLD (Fig. 3), its interaction with other PLD isozymes is unknown.

In agreement with Hammond et al. (32, 33) who studied two alternatively spliced forms of hPLD1, rPLD was stimulated by ARF, RhoA, and PKCa in vitro (Figs. 6 and 7). The enzyme was also responsive to constitutively active V14RhoA and phorbol ester in intact COS-7 cells (Figs. 4A and 5). Surprisingly, although there was evidence that constitutively active L71ARF3 activated the endogenous PLD of COS-7 cells in vivo (Fig. 4A), an unequivocal effect of this small G protein on rPLD in the intact cells could not be demonstrated (i.e. the increase in PLD activity in the cells transfected with L71ARF3 plus rPLD was inconclusive).
could be attributed to the sum of their effects when expressed alone (Fig. 4A). This cannot be ascribed to a failure of active ARF to be expressed because the protein was detected by Western blotting (Fig. 3) and stimulated the endogenous PLD in vivo (Fig. 4A). However, the possibility that L71ARF3 and rPLD were not localized in the same subcellular compartment remains, particularly because L71ARF3 was barely detectable in the membranes (Fig. 3). In this regard, membranes from cells transfected with rPLD, L71ARF3, and V14RhoA did show stimulation of PLD activity by the constitutively active forms of ARF3 and RhoA, although the magnitude of the effects was smaller than observed in vivo (Fig. 4A). Other explanations include an intrinsic insensitivity of rPLD to stimulation by ARF compared with endogenous (COS-7) PLD, a greater susceptibility of rPLD to inhibitory proteins possibly present in COS-7 cells (25–29), and a lack of responsiveness of rPLD to proteins that magnify the effect of ARF (5, 15, 20–24). In relation to these possibilities, it should be noted that the endogenous PLD of COS-7 cells differs in several respects from rPLD. For example, it does not respond to RhoA in vivo or in vitro, and it does not respond to PKCa in vitro. Furthermore, it is not recognized by several antisera raised to sequences in rPLD, hPLD1, and PLD2.

In COS-7 cells expressing rPLD in combination with wild-type ARF3 or RhoA, stimulation of PLD activity by the G protein was observed. This is probably due to presence of stimulatory agonists (e.g. lysophosphatidic acid) present in the serum added to the incubation medium. Lysophosphatidic acid induces translocation of RhoA, consistent with its activation (38). The postulated role of serum was supported by the decrease in PLD activation in serum-deprived cells (data not shown).

The observation that rPLD responds to RhoA and PKC but not to ARF in the intact cells raises questions about the physiological significance of in vitro findings with PLD preparations. For example, it is not certain how the concentrations of

**Fig. 6. Effects of mARF and RhoA on PLD in membranes (A) and cytosol (B) from COS-7 cells transfected with either vector or rPLD cDNA.** The cells were transfected and membrane and cytosol fractions were prepared as described under “Experimental Procedures.” PLD activity was assayed as described under “Experimental Procedures” by measuring the formation of [3H]PtdBut from [3H]PC incorporated into phospholipid vesicles containing PIP2 (7). The concentrations of GTPγS, mARF, and RhoA were 30, 1, and 0.1 μM, respectively. The incubations contained 3 μg of either membrane or cytosolic protein, and incubation was for 30 min at 37 °C. Data are representative of two experiments conducted in duplicate.
ARF and Rho employed in such studies relate to those in the cell, and the effects of proteins that enhance or inhibit the effects of these G proteins in vitro are also difficult to translate to the in vivo situation. However, in vitro studies do allow PLD isozymes to be characterized and differentiated and provide better definition of regulatory mechanisms.

PKCα produced a small (2-fold) but reproducible stimulation of PLD activity in COS-7 membranes (Fig. 7). The findings produced some surprises. For example, phorbol ester and PKCα alone stimulated the enzyme, implying the presence of a PKC isozyme(s) and a PKC activator(s) in the membranes. However, PKC is known to associate with membranes, and diacylglycerol can be generated in membranes through phospholipase C and D action. The greatest surprise was the fact that the effects of PMA and PKCα were observed in the virtual absence of ATP, as determined by assay, and were not enhanced by addition of the nucleotide or its nonhydrolyzable analogs (Fig. 7). Phosphorylation-independent activation of PLD by PKC isozymes has previously been reported (3–6), but there is also evidence that the activation requires ATP (2).
should be noted that the observation that PKC can activate PLD in vitro without phosphorylation does not preclude an additional ATP-dependent regulatory mechanism in vivo. Indeed, phorbol ester produced a large increase in PLD activity in vector-transfected COS-7 cells in vitro (Fig. 5), but neither PKC nor PKCa could activate the endogenous PLD of these cells in vitro. Furthermore, the fold changes induced by PMA in the intact cells (Fig. 5) were much greater than those observed in vitro (Fig. 7). These findings indicate the existence of other mechanisms by which PMA activates PLD in intact cells.

The issue of the subcellular localization of small G proteins and PLD has been alluded to above. The present study just examined cytosol and a crude membrane fraction of COS-7 cells, but rPLD was consistently found in the membranes, although it was also variably present in the cytosol (Figs. 3 and 4C). The presence of some rPLD in the cytosol was also indicated by the activity measurements shown in Fig. 6B. However, it should be cautioned that these findings with overexpressed enzyme may not reflect the distribution of PLD in normal cells, where the enzyme may be largely confined to membranes. Concerning the subcellular location of the expressed small G proteins, ARF and L71ARF3 were predominantly in the cytosol (Fig. 3), and endogenous RhoA was also almost exclusively in the cytosol, whereas there was some V14RhoA in the membranes. Because the natural substrate for PLD is membrane phospholipid, these observations suggest that membrane translocation of one or more of these G proteins would be required for activation of PLD in vivo. Agonist-induced translocation of ARF and Rho family proteins has been observed in some cell types (10, 11, 36), and there is some evidence that this is an important component of the mechanism(s) by which these proteins act (10, 11, 38, 39). Although several studies have shown that ARF, RhoA, and PKC interact synergistically to stimulate PLD activity in enzyme and membrane fractions from several tissues or cell types (5, 6, 14, 22, 40) and these proteins produce striking synergism on pure preparations of hPLD1 (33), we failed to see such synergism in intact COS-7 cells expressing these proteins (Fig. 4B) or in membranes from cells expressing rPLD and incubated with ARF, RhoA, and PKCa in vitro (Fig. 9). Although the synergism observed in previous studies with membrane fractions and partially purified PLD could be explained by the presence of other isozymes of PLD, the reason(s) for the difference between our results and those of Hammond et al. (33), who studied the human homologue of rPLD, is not clear. Comparison of the two sequences indicates substantial amino acid differences between residues 507 and 574. It could be speculated that residues in this sequence could be involved in the synergistic interactions observed with hPLD1.

A surprising observation of the present study, in agreement with the findings of Hammond et al. (32, 33), was that a single PLD isozyme could be regulated in vitro by ARF, RhoA, and PKCa. Conventional wisdom, based on findings with phosphoinositol phospholipase C and previous biochemical data (1, 30, 31), suggested that these regulatory agents would act on sepa-
arate PLD isoforms. As described above, selective regulation of PLD by these agents could occur as a result of their selective activation by agonists or their translocation to membranes enriched in certain PLD isoforms. The possibility also exists that selective inhibitors or stimulators of their actions on PLD could be very important in regulation of the enzymes. These regulatory proteins could also show specificity for certain PLD isoforms. In conclusion, the present findings indicate that the selective inhibitors or stimulators of their actions on PLD could also show specificity for certain PLD isozymes. The possibility also exists that selective regulation of PLD isozymes could be very important in regulation of the enzymes. These regulatory proteins could also show specificity for certain PLD isoforms. In conclusion, the present findings indicate that the selective inhibitors or stimulators of their actions on PLD could be very important in regulation of the enzymes. These regulatory proteins could also show specificity for certain PLD isozymes. The possibility also exists that selective regulation of PLD isozymes could be very important in regulation of the enzymes.

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