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Phospholipase D: Enzymology, Mechanisms of Regulation, and Function

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Exton, John H. Phospholipase D: Enzymology, Mechanisms of Regulation, and Function. *Physiol. Rev.* 77: 303–320, 1997.—Phospholipase D exists in various forms that differ in their regulation but predominantly hydrolyze phosphatidylcholine. The Ca^{2+}-dependent isozymes of protein kinase C regulate phospholipase D in vitro and play a major role in its control by growth factors and G protein-linked agonists in vivo. Recent studies have demonstrated that small G proteins of the ADP-ribosylation factor (ARF) and Rho families activate the enzyme in vitro, and evidence is accumulating that they also are involved in its control in vivo. Both types of G protein play important roles in cellular function, and the possible mechanisms by which they are activated by agonists are discussed. There is also emerging evidence of the control of phospholipase D and Rho proteins by soluble tyrosine kinases and novel serine/threonine kinases. The possible role of these kinases in agonist regulation of phospholipase D is discussed. The function of phospholipase D in cells is still poorly defined. Postulated roles of phosphatidic acid produced by phospholipase D action include the activation of Ca^{2+}-independent isoforms of protein kinase C, the regulation of growth and the cytoskeleton in fibroblasts, and control of the respiratory burst in neutrophils. Another important function of phosphatidic acid is to act as a substrate for a specific phospholipase A_2 to generate lysophosphatidic acid, which is becoming increasingly recognized as a major intercellular messenger. Finally, it is possible that the phospholipid changes induced in various cellular membranes by phospholipase D may per se play an important role in vesicle trafficking and other membrane-associated events.

I. INTRODUCTION

Phospholipase D (PLD) is a ubiquitous enzyme that hydrolyzes phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline. Phospholipase D activity toward phosphatidylethanolamine (PE) and phosphatidylinositol (PI) has also been reported. Phosphatidylcholine-PLD is regulated by a great variety of hormones, neurotransmitters, growth factors, cytokines, and related molecules involved in intercellular communication. For this reason, it is believed to play a role in signal transduction in many cell types. The signaling molecule produced by PLD is generally considered to be PA, since resting levels of choline in most cells are high. Thus activation of PLD in cells usually results in a severalfold increase in PA, but may not cause a detectable rise in intracellular choline.

The products of the metabolism of PA are 1,2-diacylglycerol (DAG), which is produced by the action of phosphatidate phosphohydrolase (PAP), and lysophosphatidic acid (LPA), which is produced by the action of a specific phospholipase A_2 (PLA_2). Many functions for PA have been proposed, namely, promotion of mitogenesis in fibroblasts, stimulation of the respiratory burst in neutrophils, increases in cell Ca^{2+}, and activation of specific protein kinases and phospholipases. In addition, much of the DAG that accumulates in stimulated cells arises from PA due to the action of PAP. Because DAG is the major cellular regulator of protein kinase C (PKC), an important role for PC hydrolysis is activation of certain isoforms of

this enzyme. Lysophosphatidic acid is a major product of PA metabolism in platelets and is now recognized to be an important intercellular signal. Many cells respond to LPA through a membrane receptor that is coupled to G proteins.

In summary, activation of PLD is elicited by a wide variety of agonists in a diversity of cell types. It produces primarily and secondarily three important lipid messengers, namely, PA, DAG, and LPA, which affect many significant cellular processes. These lipids are also components of the biosynthetic pathways for triacylglycerol and phospholipids. However, these pathways are mainly localized to the endoplasmic reticulum and are controlled by different mechanisms.

II. ENZYMOLOGY OF PHOSPHOLIPASE D

For many years, PLD was considered to be absent from animal tissues. However, Saito and Kanfer (187) first demonstrated its presence in rat brain. Soluble preparations of the enzyme from brain released choline and ethanolamine from PC and PE, respectively. They also catalyzed exchange of these bases into the corresponding phospholipids. An important further discovery was that PLD catalyzed the transphosphatidylation reaction whereby the phosphatidyl group of the phospholipid is transferred to glycerol or a primary alcohol such as ethanol, propanol, or butanol (90). This reaction is now regarded as unequivocal evidence of PLD activity.

The two substrates of PLD, PC and PE, contain significant amounts of ether-linked species, and the enzyme can hydrolyze both the alkyl-linked and acyl-linked forms of the two phospholipids (35, 67, 75, 117, 144). Interestingly, when the enzyme in Madin-Darby canine kidney cells was activated by phorbol ester, there was preferential hydrolysis of alkyl-PC, whereas the G protein-regulated PLD preferred acyl-PC (35, 75). This difference was not seen in other cells (67, 117), but it suggests the existence of PLD isozymes that differ in their substrate specificity. Any differential action of PLD on alkyl- and acyl-phospholipids would yield different amounts of alkylacylglycerol and DAG through the action of PAP. Because these have different effects on PKC (23, 35), any selectivity of PLD isozymes for the two phospholipid subclasses would have functional consequences.

Phospholipase D activity has been detected in all mammalian tissues examined (10, 13, 47, 171), but efforts to purify the enzyme have met with limited success. On the basis of the properties of partially purified preparations of PLD, there appear to be several isozymes. The PLD that is activated by agonists acts mainly on PC (13, 47), although there have been reports of the hydrolysis of PE also (101, 102). Based on its properties and substrate specificity, the enzyme differs from that which hydrolyzes the glycosyl PI anchor for many surface proteins and that which acts on PI (13).

Phospholipase D activity has been found in the plasma membranes and cytosol of most tissues, and also in the endoplasmic reticulum, Golgi, and nuclei of certain cells and tissues (13, 171). The enzyme has been partially purified from rat brain microsomes using Miranol and Triton detergents (104, 201) and completely purified from porcine lung microsomes using heptylthioglucoside and seven chromatographic steps (157). The enzyme from lung had an M_r of 190,000 and was specific for PC. It was not dependent on Ca^{2+} or Mg^{2+}, but was stimulated by these ions. Like the brain enzyme, it was activated by unsaturated fatty acids. However, it is not known whether it is subject to control by PKC or by small or heterotrimeric G proteins. Brown et al. (21) have solubilized PLD from HL-60 cell membranes using NaCl. The enzyme was potently activated by phosphatidylinositol 4,5-bisphosphate (PIP_2) and by the small G protein ADP-ribosylation factor (ARF). In further work, Brown et al. (20) partially purified PLD from porcine brain membranes. Like the HL-60 enzyme, its activity was markedly enhanced by PIP_2 and ARF. However, the stimulation by ARF was increased further by the addition of undefined cytosolic fractions (194). The enzyme had an M_r of 95,000 as determined by hydrodynamic analysis (20).

Human placenta contains membrane-bound and cytosolic PLD activities that are both stimulated by the small G proteins ARF and RhoA (218). Partial purification resulted in loss of the RhoA effect but retention of the ARF effect. The purified membrane-associated and cytosolic enzymes were both stimulated by PIP_2 and inhibited by oleate. The presence of two forms of PC-PLD in rat brain membranes has been demonstrated by Massenburg et al. (138) using Triton solubilization and heparin sodium chromatography. One form was dependent on oleate for activity, whereas the other was markedly activated by ARF and PIP_2. Soluble forms of PLD have been identified in eosinophils (93), HL-60 cells (193), and several mammalian tissues (171, 219).

An ARF-responsive human PLD has recently been cloned by screening a HeLa cDNA library (62). It has an M_r of 120,000 and has many residues that are conserved in plant (rice, corn, and castor bean) and yeast (*Saccharomyces cerevisiae*) PLDs, but no recognizable domains (SH2, SH3, or PH). There are six sequences that are identical in yeast and human PLD. Expression of the human PLD in Sf9 insect cells resulted in a large enhancement of cytosolic and membrane PLD activity that was selective for PC and catalyzed transphosphatidylation (62). The expressed enzyme was activated by PIP_2, but inhibited by oleate. It was also stimulated markedly by ARF when expressed in either Sf9 or COS-7 cells. In a followup study, PLD was shown to occur in two alternatively spliced forms, one of which lacks a 38-amino acid sequence (62a).

Both forms, when expressed in Sf9 cells and purified to homogeneity, were strongly activated by PIP_2, PIP_3, and ARF, and to a lesser extent by members of the Rho family of small G proteins (RhoA, Rac1, CDC42). Protein kinase C-α also activated both isoforms in the absence of ATP, and its effect was synergistic with ARF and the Rho proteins (62a). These data indicate that the three major regulators of PLD (ARF, Rho proteins, and PKC-α) directly activate both PLD isoforms by interacting at different sites on the enzymes.

III. REGULATION OF PHOSPHOLIPASE D

As described in section I, a very large number of agonists increase the activity of PLD in many cells and tissues (13). Because many of these agonists act through receptors coupled to the heterotrimeric G proteins G_q and G_i, it is clear that the signal(s) arising from activation of these G proteins must be transmitted to PLD isozymes. Likewise, many of the agonists are growth factors that act through receptors with tyrosine kinase activity. Thus signals arising from this activity or from the changes due to autophosphorylation of the receptors must also be communicated to PLD isozymes. As described in section IV, there is much evidence that PKC mediates the effects of many agonists on PLD, since most receptors coupled to G_q and G_i or possessing tyrosine kinase activity are capable of inducing significant PIP_2 hydrolysis and PKC activation. However, there is also evidence that PKC activation cannot entirely explain the actions of these agonists on PLD, implying the existence of other mechanisms.

IV. ROLE OF PROTEIN KINASE C IN ACTIVATION OF PHOSPHOLIPASE D

Tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) stimulate PLD in a large number of tissues and cells (10, 13, 46, 47). This indication that the enzyme is regulated by PKC is supported by many studies employing PKC inhibitors and downregulation of the enzyme. Many agents have been employed to inhibit PKC. Some lack specificity insofar as they inhibit other protein kinases or affect other enzymes [e.g., 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), staurosporine, and sphingosine]. Nevertheless, studies with a variety of these inhibitors (H-7, staurosporine, sphingosine, 1-*O*-hexadecyl-2-*O*-methyl glycerol, sangivamycin, calphostin C, bisindolylmaleimide, chelerythrine, and Ro-31–8220) generally support the involvement of PKC in the actions of many G protein-linked agonists and growth factors on PC-PLD (6a, 9, 30, 56, 57, 72, 96, 166, 172, 210, 212, 225). On the other hand, negative or partial effects have been observed in some studies (91, 126, 129, 132, 211). Thus the findings with PKC inhibitors support the partial or complete dependence of agonist regulation of PLD on PKC in many cell types. However, it may be recognized that there are also studies in which PLD and PKC activities are dissociated or in which PKC inhibitors have greater effects on the stimulation of PLD by phorbol esters than by agonists.

Except for a few reports, downregulation of PKC has been found to completely or partly inhibit the activation of PLD by a variety of growth factors and G protein-linked agonists (13). For example, prolonged treatment of cells with PMA or phorbol dibutyrate has been shown to completely block the ability of G protein-linked agonists such as angiotensin II, bradykinin, bombesin, carbachol, LPA, gonadotropin-releasing hormone, vasopressin, endothelin, thyrotropin-stimulating hormone, and prostaglandin $F_{2\alpha}$ to activate PC-PLD in a variety of cell types (9, 32, 56, 126, 131, 135, 136, 151, 170, 210, 212, 217). Likewise, this treatment abolished or attenuated the PLD response to growth factors, e.g., epidermal growth factor and platelet-derived growth factor (PDGF), in most instances (9, 92, 168, 170, 225). In summary, the results of the downregulation and inhibitor studies indicate that agonist activation of PLD involves both PKC-dependent and -independent mechanisms.

There is much evidence that the PKC-dependent mechanism involves the initial hydrolysis of PIP_2 to yield inositol trisphosphate and DAG, since the agonists that activate PLD also activate phosphoinositide phospholipase C (PI-PLC) (13). In some cases where PC hydrolysis has been reported to occur in the absence of PIP_2 breakdown, further analysis has shown a small, but significant, production of inositol phosphates (225). The small response is due to its downregulation by a PKC-mediated mechanism. When PKC inhibitors are present, the accumulation of inositol phosphates is enhanced (225).

A clear example of the involvement of PI-PLC in the activation of PLD by growth factors comes from the work of Yeo et al. (226). Using TRMP cells (a kidney epithelial cell line) expressing wild-type or mutant PDGF receptors, these workers (226) showed that receptors with mutation of Tyr-1021 to Phe, which results in a loss of activation of PI-PLC-γ, did not mediate PDGF activation of PLD. On the other hand, retention of Tyr-1021, but mutation of other Tyr residues involved in signaling, permitted PLD activation (226). Furthermore, treatment of the cells with the PKC inhibitor Ro-31–8220 or downregulation of PKC by phorbol ester treatment greatly impaired the PLD response. These results indicate that PLD activation by PDGF in these cells is largely, if not entirely, dependent on PI-PLC and PKC. This conclusion receives support from the work of Lee et al. (120), who found that NIH 3T3 fibroblasts that stably overexpress PI-PLC-γ1 have a greatly enhanced PLD response to PDGF and that treatment with the tyrosine kinase inhibitor genistein greatly decreased PLD activity and also tyrosine phosphorylation

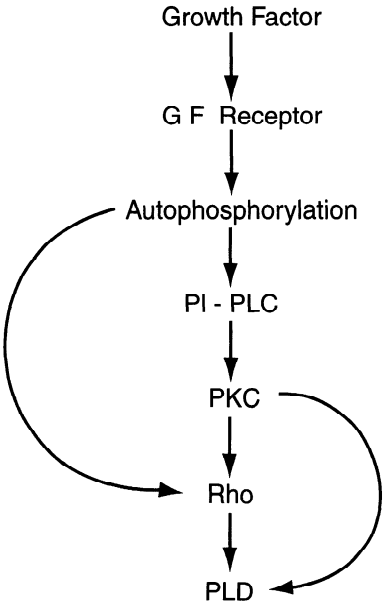

FIG. 1. Postulated mechanisms of activation of phospholipase D (PLD) by growth factors. Pathway of activation of PLD mediated by protein kinase C (PKC) is shown. It is not known whether PKC interacts directly with PLD or via Rho or another protein. Growth factors activate Rho by a separate pathway, which probably involves multiple steps. Rho and PKC may interact synergistically to activate PLD. Relative utilization of pathways may vary with different agonists and different cell types. GF, growth factor; PI, phosphatidylinositol; PLC, phospholipase C.

of the PDGF receptor and PI-PLC-γ1 in cells stimulated with PDGF. Downregulation of PKC by PMA pretreatment also caused complete inhibition of PLD activation by PDGF. Figures 1 and 2 illustrate the postulated roles of PKC and small G proteins in the regulation of PLD by growth factors and agonists linked to heptahelical receptors that activate G_q or G_i/G_o.

In another approach to determining the role of PKC in PC-PLD regulation, specific PKC isozymes have been overexpressed in fibroblasts. These experiments have shown an enhancement of PLD activation by endothelin, thrombin, and PDGF in cells overexpressing the α- and β_1-isozymes (44, 161, 162). On the other hand, depletion of PKC-α by antisense methods decreased the activation of the phospholipase by purinergic agonists (6a). Thus these studies support other evidence that Ca^{2+}-dependent PKC isozymes play an important role in the regulation of PC-PLD.

Regulation of PLD by PKC could be direct or involve another protein(s). A direct effect of PKC-α on homogeneous preparations of PLD has recently been described (62a), and there have been several reports that addition of PKC to plasma membranes enhances the membrane-associated PLD activity (28, 130, 155, 156). Although one of these studies provided evidence for a phosphorylation-dependent mechanism (130), the others indicated that phosphorylation was not involved in the effect. For example, the activation of PLD by the kinase occurred in the demonstrated absence of ATP (28) and was dissociated from the phosphorylation activity of the kinase (28, 155). The PKC activation of membrane PLD showed a concentration dependence on both phorbol ester and Ca^{2+} (28, 29). This indication that conventional Ca^{2+}-dependent PKC isozymes were involved was supported by experiments testing individual isozymes, which showed that only the α, βI, and βII forms were effective (29, 156).

In studies of the regulation of PLD partially purified from porcine brain, Singer et al. (194) identified a factor that activated the enzyme in a nucleotide-independent manner. This factor was later shown to be PKC-α (195). In accordance with the findings of Conricode et al. (28), the activation of PLD by this PKC isozyme was enhanced by phorbol ester and Ca^{2+}, but occurred in the absence of ATP. Protein kinase C-α was shown to interact synergistically with the small G proteins ARF and RhoA to activate PLD. The synergism was marked and was also seen when ARF and RhoA were combined. Very similar findings have recently been reported by Hammond et al. (62a) using homogeneous PLD. Cleavage of PKC-α with trypsin showed that the PLD stimulatory activity resided in the regulatory domain. Furthermore, staurosporine and protein phosphatase treatment inhibited the kinase activity of the enzyme without altering its effect on PLD. The results of Singer et al. (195) therefore strongly support a mechanism of activation of PLD by this PKC isozyme that

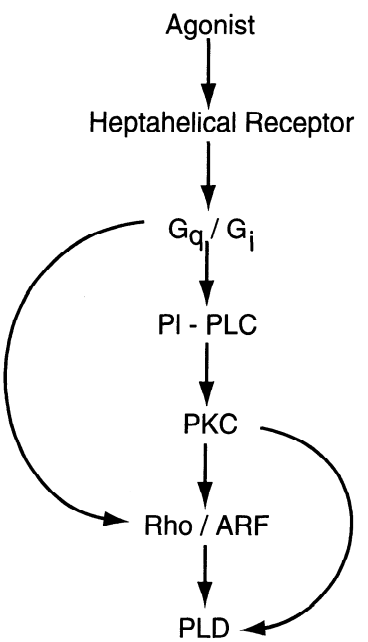

FIG. 2. Postulated mechanisms of activation of PLD by agonists that interact with heptahelical receptors (i.e., receptors with 7 transmembrane segments). Pathway of activation of PLD mediated by PKC is shown. It is not known whether PKC interacts directly with PLD or via Rho, ADP-ribosylation factor (ARF), or another protein. Members of G_q and G_i families activate Rho and/or ARF by alternative pathways, which probably involve multiple steps. Rho and ARF may interact synergistically with PKC to activate PLD. Relative utilization of pathways may vary with different agonists and different cell types.

is independent of phosphorylation. Ohguchi et al. (156) also used chromatographic procedures to show that a cytosolic factor required for the activation of PLD in HL-60 membranes by phorbol ester or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was PKC. Although MgATP was required, this was attributed to its stimulation of the resynthesis of PIP_2, which is required for PLD activity. The α- and β-isozymes of PKC were able to stimulate PLD, but not the γ-isozyme. Protein kinase C-α plus phorbol ester were also found to markedly enhance the stimulation of PLD by GTPγS plus RhoA. As noted above, there are other reports indicating synergistic interactions between PKC and small G proteins in the regulation of PLD.

V. ROLE OF CALCIUM IONS IN REGULATION OF PHOSPHOLIPASE D

Studies with the Ca^{2+} ionophores A23187 and ionomycin indicate that a rise in cytosolic Ca^{2+} can stimulate PLD (13). There is also evidence that depletion of cellular Ca^{2+} by the chelators ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid and 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid results in inhibition of the stimulation of the enzyme by agonists linked to the heterotrimeric G proteins G_q and G_i (6a, 61, 73, 95, 125, 224). Activation of the P_{2z} purinergic receptor/ion channel by ATP in leukemic lymphocytes has also been shown to activate PLD, apparently via Ca^{2+} influx (50). A major question is whether these findings stem from the involvement of Ca^{2+}-dependent PKC isozymes or indicate another role for Ca^{2+} in PLD activation. To date, there have been no reported studies of the role of Ca^{2+} in the activation of the phospholipase by growth factors. Studies of the effects of Ca^{2+} on partially purified preparations of PLD from various sources have usually shown stimulation, but the concentrations of the cation required for stimulation have varied widely, and high concentrations have generally been inhibitory (13, 20, 27, 157, 193, 201). Thus the possibility that there is direct physiological control of PLD by changes in cytosolic Ca^{2+} is not yet supported.

VI. ROLE OF SMALL G PROTEIN ADP-RIBOSYLATION FACTOR IN REGULATION OF PHOSPHOLIPASE D

A new development in the study of the regulation of PLD is the involvement of small G proteins of the ARF and Rho families. This arose from early studies showing that cytosol was required for the stimulatory effect of GTPγS on PLD in membranes of neutrophils or HL-60 cells (2, 160). A similar conclusion was drawn from experiments with permeabilized HL-60 cells, in which the ability of GTPγS to stimulate the enzyme declined with time as cytosolic proteins leaked from the cells (51, 52). In these experiments, activation of PLD could also be restored by adding brain cytosol, and the reconstituting factor was demonstrated to be a protein of ~16-kDa mass (52). In simultaneous studies, Brown et al. (21) and Cockcroft et al. (27) purified the protein to homogeneity and showed that it had amino acid sequences corresponding to ARF1 and ARF3. Both groups showed that recombinant ARF was effective and that myristoylation greatly enhanced its potency (20, 21, 27). Activation of PLD has been observed with myristoylated ARF1, -3, -5, and -6, with little difference in their relative potency (20, 138). Importantly, Hammond et al. (62a) have shown that ARF can directly interact with some isoforms of the enzyme.

Although it is now well accepted that ARF is required for the activation of PLD in neutrophils and HL-60 cells by GTP analogues, more recent evidence indicates the requirement for additional cytosolic factors (17, 115, 194, 195). Two groups have identified one of these factors as a 50-kDa protein (17, 115), but its structure and function remain unknown, i.e., whether it interacts with PLD or with ARF. Another factor has been shown to be the α-isozyme of PKC (195). As noted above, this kinase can activate the enzyme directly or synergize with ARF or Rho to produce a marked activation (195).

Houle et al. (70) have observed that translocation of ARF from the cytosol to membranes is associated with activation of PLD in HL-60 cells. Thus, in membranes from cells treated with f-Met-Leu-Phe or phorbol ester, the response of PLD to GTPγS was enhanced, and Western blotting revealed an increase in membrane-associated ARF. Both effects were enhanced by prior treatment of the cells with cytochalasin B. The findings support the association of ARF with the membrane as a necessary step for activation of PLD.

ADP-ribosylation factor was originally discovered as a factor required for the ADP-ribosylation of $G_s\alpha$ by cholera toxin but was later shown to be involved in protein trafficking in the Golgi (149, 150). Interestingly, the domain of ARF involved in activating PLD is different from that involved in cholera toxin activation (227). Although most studies of the regulation of PC-PLD have utilized preparations of plasma membranes and cytosol, the enzyme is also present in Golgi membranes, where it is activated by ARF in a brefeldin A-sensitive manner (107, 171). Brefeldin A is an inhibitor of the activation of ARF by GTP/GDP exchange (38, 64, 177).

The majority of investigations of the regulation of PLD by ARF have been carried out in vitro. However, there have been some studies indicating that this regulation occurs in intact cells. Using human embryonic kidney (HEK) cells stably expressing M3 muscarinic receptors, Rümenapp et al. (186) found that the activation of PLD by carbachol was inhibited by brefeldin A in a dose-dependent manner. On the other hand, the compound did not block the activation of

PLD by GTPγS in digitonin-permeabilized cells, presumably because activation of ARF by this nucleotide is not dependent on a nucleotide-exchange factor (GDP-dissociation stimulatory protein). Interestingly, GTPγS inhibited the release of ARF from the cells caused by permeabilization. This effect was associated with the translocation of ARF from cytosol to membranes. Such a translocation was also observed in cells treated with carbachol before permeabilization (186). In addition to indicating a role for ARF in the activation of PLD by a G protein-linked agonist, these results suggest that activation of ARF through an increase in its GTP binding is associated with its membrane translocation.

In unpublished studies, I. Guillemain-Radassi and J. Exton have found that treatment of HL-60 cells with brefeldin A inhibits the activation of PLD by f-Met-Leu-Phe, ATP, and PMA, but does not alter the effect of GTPγS on the enzyme in permeabilized cells. These results thus resemble those of Rumenapp et al. (186) and support a role for ARF in the regulation of PLD in vivo. Figure 2 illustrates the postulated role of ARF in the regulation of PLD by agonists that activate G_q or G_i/G_o.

VII. ROLE OF SMALL G PROTEINS OF THE RHO FAMILY IN REGULATION OF PHOSPHOLIPASE D

In a study of the effects of GTPγS on the PC-PLD activity of neutrophil lysates, Bowman et al. (18) found that protein factors in both the cytosol and membranes were needed for activity. Contrary to expectations, the GTPγS-binding protein was found to be associated with the membranes, and its properties indicated that it was a small G protein rather than a heterotrimeric G protein. This was supported by the fact that a nonspecific GDP dissociation stimulatory protein (GDS) for small G proteins increased the ability of GTP to stimulate the membrane-associated PLD. When a GDP dissociation inhibitor (GDI) that was selective for members of the Rho family was tested, it was found to inhibit the activation of the enzyme by GTPγS (18).

The findings of Bowman et al. (18) have been confirmed using membranes or homogenates from HL-60 cells, neutrophils, or rat liver. Thus the stimulation of PLD by GTPγS was inhibited by Rho-GDI in the membranes (114, 134, 155, 193) and in the homogenates (17). Furthermore, the addition of recombinant RhoA, Rac1, or CDC42 to membranes previously treated with Rho-GDI to extract Rho family proteins resulted in restoration of GTPγS stimulation of the phospholipase (114, 134, 193). In the case of HL-60 membranes, pretreatment with Rho-GDI was not necessary (156, 193). RhoA was the most effective Rho family member for PLD activation in both HL-60 and liver membranes. As expected, ARF was also effective in promoting the stimulation of PC-PLD in HL-60 membranes, and the combination of ARF and RhoA produced a synergistic effect (193). Synergism between these two families of small G proteins in the activation of PLD has also been reported by Singer and co-workers (194, 195) and Kuribara et al. (112).

A surprising recent finding has been the observation that homogeneous preparations of two splice variants of PLD are activated to a similar extent and with similar potency by RhoA, Rac1, and CDC42 (62a). Combination of these Rho family proteins did not increase the PLD activity beyond that seen with one protein alone, suggesting that they interact with PLD through a common domain. Because the different Rho family proteins produce diverse cellular effects, their very similar action on PLD is unexpected. However, it is known that they have diverse effectors and are differentially activated by agonists (153, 200).

Although in some studies, addition of Rho proteins alone to membranes is capable of inducing GTPγS-stimulated PLD activity (114, 134, 155, 193), it was found that the effect of RhoA on the PLD activity of neutrophil membranes was greatly enhanced by the addition of a 50-kDa protein partially purified from neutrophil cytosol. This suggestion that an additional protein is required for the action of RhoA on PLD is supported by the findings of Vinggaard et al. (218), who observed that, after partial purification, PLD from human placenta lost its response to RhoA while retaining its activation by ARF. Because a Rho-responsive PC-PLD has not been purified or cloned, it is not known whether or not Rho interacts with the enzyme directly. Furthermore, the role of the 50-kDa protein is unknown. Although it is very likely that the ARF-responsive and Rho-responsive PLDs are different isozymes, this is not clearly established at present.

Han et al. (63) observed that rat brain cytosol contains proteins that markedly inhibit PLD stimulated by ARF or PIP_2. Two of these proteins were purified to homogeneity and shown to have apparent molecular masses of 150 and 135 kDa. The 150-kDa protein blocked the activation of PLD by ARF, RhoA, and Cdc42 and was effective at subnanomolar concentrations. Further studies suggested that the inhibitor does not act by competing with the activators or substrates for PLD. Geny et al. (53) have also reported the identification of a brain cytosolic protein that inhibits ARF-dependent PLD activity, but this has a much higher molecular mass. A key question is what role these inhibitors play in the regulation of PLD in vivo.

As alluded to in section IV, many reports have shown a synergism between PMA and GTPγS in the activation of PLD in membranes from several tissues (33, 51, 155, 160, 173). Interestingly, more recent evidence indicates that the synergism involves PKC and both Rho and ARF. Thus Ohguchi et al. (155, 156) observed that Rho-GDI inhibited the stimulatory effect of PKC on HL-60 membrane PLD activity and that the addition of RhoA plus PKC-α produced a synergistic activation in the presence

of phorbol ester and GTPγS. Working with a partially purified PLD preparation from brain, Singer et al. (195) showed that PKC-α produced a synergistic activation when added with either ARF or RhoA. The mechanisms involved in the interactions of PKC with the two small G proteins are undefined.

A key issue concerning the role of ARF and Rho family members in the regulation of PLD is how these small G proteins fit into the general scheme by which agonists control this enzyme in intact cells. In addition to the evidence presented in section VI, recent studies have also indicated that Rho family members are involved in the signaling pathways initiated by receptors linked to G proteins and those with tyrosine kinase activity and are also involved in the regulation of PLD in intact cells. There is much evidence that the Rho family member Rac mediates the effects of growth factors on actin polymerization (membrane ruffling or formation of lamellipodia) in fibroblasts (153, 180, 182) and that another member Cdc42Hs is involved in filopodia and actin microspike formation induced by bradykinin in Swiss 3T3 cells (106, 154). Furthermore, Rho is involved in the effects of the G protein-linked agonists LPA and bombesin on the formation of actin stress fibers and the assembly of focal adhesions in fibroblasts (163, 181, 200). Thus microinjection of constitutively active mutated forms of Rho (V14 Rho) and of Rac (V12 Rac) reproduced the specific effects of LPA and PDGF, respectively, on actin polymerization (143, 154, 163, 182). Furthermore, injection of a dominant negative form of Rac (N17 Rac) blocked the effects of growth factors (182), and treatment of Rho with the C3 exoenzyme of *Clostridium botulinum* to ADP-ribosylate and inactivate it resulted in the generation of a dominant negative form of Rho that inhibited the effects of LPA on actin polymerization (181). Disaggregation of actin filaments was also seen with the B toxin of *Clostridium difficile*, which glucosylates and inactivates Rho proteins (88). These results complement those obtained when the C3 exoenzyme was injected directly into fibroblasts and other cells, which resulted in marked morphological changes and an inhibition of the cytoskeletal responses to LPA, endothelin, bombesin, or thrombin (24, 81, 163, 178, 181, 185).

In addition to the preceding evidence that Rho and Rac mediate some of the cytoskeletal changes induced by G protein-linked agonists and growth factors, there are data strongly implicating their involvement in the regulation of PLD. Schmidt et al. (189) have reported that treatment of HEK cells expressing the M3 muscarinic receptor with *C. difficile* toxin B blocks carbachol stimulation of PLD. The toxin also inhibits activation of the enzyme by GTPγS and AlF_4^- in intact or permeabilized cells. Malcolm et al. (133) have also shown that scrape-loading of C3 exoenzyme into Rat1 fibroblasts greatly attenuates the activation of PLD by LPA, endothelin-1, and phorbol ester without altering the effect of the agonists on PI-PLC. The effect was not due to disruption of the actin skeleton, since it was not mimicked by cytochalasin D. Activation of PLD by agonists was associated with translocation of RhoA to membranes. In unpublished studies of the effects of PDGF and EGF on PLD in fibroblasts, J. Hess, A. Ross, and J. Exton also found inhibition of the PDGF effect by C3 exoenzyme. They also showed that the effect of EGF on PLD was greatly enhanced in fibroblasts overexpressing Rac1 and abolished in cells expressing dominant negative N17Rac1. These studies therefore indicate a role for Rho in the activation of PLD by agonists linked to G proteins and PKC, and a role for both Rho and Rac in the activation induced by growth factors. On the basis of other data (181), Rho appears to be downstream from Rac. Figures 1 and 2 illustrate the postulated role of Rho in the regulation of PLD by growth factors and agonists linked to G_q or G_i/G_o.

Because the above results indicate that growth factors are able to activate Rac and Rho, and G protein-linked agonists can activate Rho in fibroblasts, these agonists must be capable of controlling one or more of the proteins that regulate the activation state (GTP/GDP) of these small G proteins. Nobes and Hall (154) have shown that wortmannin, an inhibitor of phosphatidylinositol 3-kinase, blocks the membrane ruffling induced in Swiss 3T3 cells by PDGF, but not that due to microinjected V12Rac1. It also does not alter focal adhesion or stress fiber formation induced by LPA, bombesin, or microinjected V14RhoA. On the other hand, the tyrosine kinase inhibitor tyrphostin inhibits the effects of LPA, but not those of activated RhoA. These data indicate that phosphatidylinositol 3-kinase is involved in the activation of Rac by growth factors, whereas tyrosine phosphorylation mediates the activation of Rho by LPA. Tyrosine kinase activity may also be needed for formation of stress fibers and focal adhesions induced by LPA, bombesin, or microinjected V14RhoA, since genistein blocked these responses (181). Rozengurt and co-workers (178, 190) have reported that C3 exoenzyme blocks the tyrosine phosphorylation of focal adhesion kinase and paxillin induced by bombesin, endothelin, or GTPγS in Swiss 3T3 cells, supporting a role for tyrosine kinase in downstream signaling from Rho.

Fleming et al. (49a) have observed that treatment of Swiss 3T3 cells with LPA or endothelin-1 causes a rapid translocation of RhoA from the cytosol to a membrane fraction. This is not induced by PDGF or accompanied by translocation of Rac1. Its relationship to the presumed activation of RhoA by these G protein-linked agonists or to the action of RhoA is being defined. The idea that the translocation is controlled by membrane-associated GDS proteins is supported by the findings of Bokoch et al. (15) and Fleming et al. (49a). They have shown that in membranes plus cytosol, GTP analogues induce the rapid membrane association of Rho, Rac, and Cdc42 and that

this is associated with dissociation of the Rho proteins from their complexes with GDI.

There is evidence of coupling between the Ras and Rho signaling pathways. For example, some groups have reported that the transformation of cells by oncogenic Ras is critically dependent on Rac or Rho (99, 169, 174, 175). This has been demonstrated by the effects of dominant negative N19Rho or N17Rac1 to inhibit the effects of oncogenic Ras and of the ability of constitutively active V14Rho to augment the effect of active Rac or active Raf (RafCAAX) to induce focus formation. In the case of constitutively active V12Rac1, this alone can cause malignant transformation, but can also synergize with active Raf (175). Examination of the changes in actin polymerization in these studies indicated that the effects of Ras on transformation were mediated by two pathways, one involving Raf and the MAP kinase cascade, and the other involving Rac and Rho, with the latter also controlling the actin cytoskeleton (99, 169, 174). A role for Rho, Rac, and Cdc42 in control of the cell cycle and DNA synthesis has been shown by the injection of constitutively active forms of these proteins into Swiss 3T3 cells (159). The effects of injection of C3 exoenzyme or expression of dominative negative Rac or Cdc42 also indicate a need for Rho family members for cell cycle progression (159). In support of the conclusions of Qiu et al. (174) and Prendergast et al. (169), Olson et al. (159) found that the active forms of Rho, Rac, and Cdc42 did not activate mitogen-associated protein (MAP) kinase, but that Rac and Cdc42 activated Jun kinase.

The mechanism of coupling between the Ras and Rho signaling pathways is not well understood. However, Rac activation by growth factors is blocked by antibodies to Grb2, suggesting that this adaptor protein, which binds son-of-sevenless (SOS)a GDS for Ras, is involved (139). Association of Rho with the PDGF type β receptor is also induced in fibroblasts by treatment with PDGF (231), suggesting that Rho may be a component of the complex of signaling proteins formed when the receptor is activated. In addition, expression of the NH_2-terminal domain of Ras-GAP that produces a complex with $p190^{RhoGAP}$ (140) has been observed to disrupt actin stress fibers and focal adhesions in Rat2 cells, consistent with impairment of Rho function. Previous work has shown that $p190^{RhoGAP}$ is tyrosine phosphorylated in cells in response to growth factors and that it associates with the SH_2-SH_3-SH_2 domain of RasGAP (45).

VIII. ROLE OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE IN REGULATION OF PHOSPHOLIPASE D

Brown et al. (21) first discovered that mammalian PLD was potently stimulated by PIP_2. Phosphatidylinositol 4-phosphate (PIP) had ~10% of the activity of PIP_2, whereas phosphatidylserine was nonstimulatory. Many other studies have confirmed the effect of PIP_2, and it is now generally included in in vitro assays of the enzyme. The effect is seen with 0.05 mol% PIP_2 and is mimicked by phosphatidylinositol 3,4,5-trisphosphate, with slightly lower potency (62a, 128). Phosphatidylinositol and PA are completely or nearly ineffective. Neomycin, which binds phosphoinositides, inhibits membrane-bound but not detergent-solubilized or partially purified PLD, and the addition of PIP_2 to purified PLD restores its sensitivity to neomycin (128). These findings support the view that PIP_2 is a required cofactor for membrane PLD activity. Furthermore, PIP_2 is required for the stimulatory effects of PKC or the small G proteins ARF and Rho on PLD (20, 21, 112, 138, 156, 171).

An important issue is whether or not PLD is regulated by changes in PIP_2 in vivo. Pertile et al. (164) have provided evidence for this in permeabilized U937 cells. In these cells, the activation of PLD by GTPγS was greatly potentiated by MgATP, which maintained elevated levels of PIP and PIP_2 in the cells. When an inhibitory antibody to phosphatidylinositol 4-kinase was introduced, PIP and PIP_2 levels fell, and there was a coincident decline in the activation of PLD by GTPγS or phorbol ester. A similar inhibition of PLD activation was observed with neomycin. Although these experiments did not explore the effects of regulating PIP_2 levels by physiological means, they support the dependence of PLD activity on PIP_2.

Exposure of cells to agonists that activate PI-PLC causes a rapid decline in PIP_2, but the level returns toward normal due to its rapid resynthesis from PI via PIP (4, 197). There is evidence that agonists and GTP analogues can activate PIP 5-kinase (197), and it is possible that the effect is mediated by the small G proteins of the Rho family (25, 179, 207). In lysates of Swiss 3T3 cells, GTPγS was observed to stimulate this kinase, and the effect was blocked by the C3 exoenzyme of *C. botulinum*, implying the involvement of Rho (25). Guanosine 5′-triphosphate-bound Rho was also effective in stimulating the enzyme, whereas GDP-Rho and GTP-Rac were less potent or ineffective. Rho family proteins were also found to bind to PIP 5-kinase in rat liver cytosol (207), but the binding was much greater with Rac1 than RhoA or Cdc42 Hs, contrary to what was found for activation of the enzyme in Swiss 3T3 cells. In further work with lysates from the latter cells, Ren et al. (179) found that Rho bound significant amounts of PIP 5-kinase, but that the association was independent of whether Rho was bound to GTPγS or GDP. Phosphatidylinositol 4-phosphate 5-kinase can also be activated by PA (84, 148), but the isozyme that is sensitive to PA is different from that which responds to Rho (179). Several attractive hypotheses have been developed concerning the roles of Rho and PA in the regulation of PIP 5-kinase and the interrelated regulation of PLD and PIP 5-kinase (128, 164). Specifically, both enzymes can be regulated by Rho; the product of PLD (PA) can regulate PIP 5-kinase, and the product of PIP 5-kinase (PIP_2) can regulate PLD.

Other factors involved in the regulation of PIP_2 synthesis are the PI transfer proteins (PI-TPs) (127). In addition to their role in the transfer of PI and PC from donor to acceptor membranes (223), these proteins may act by presenting PIP and PIP_2 to the enzymes that utilize them, e.g., PI 4-kinase and PI-PLC (34, 94, 127, 203). Kauffmann-Zeh et al. (94) have provided evidence for the physical association of PI-TP and PI 4-kinase and PI-PLC-γ in cells treated with epidermal growth factor. The roles of PI-TP, PI-kinases, and PLD in vesicle trafficking in Golgi and endosomes are discussed elsewhere (39, 89, 127, 150).

IX. ROLE OF SOLUBLE TYROSINE KINASES AND NOVEL SERINE/THREONINE KINASES IN REGULATION OF PHOSPHOLIPASE D

As noted in section III, growth factors whose receptors possess intrinsic tyrosine kinase activity are able to activate PLD. Activation of the T-cell antigen receptor and of the Fc_ϵ receptor for immunoglobulin E of mast cells also causes activation of PLD (37, 125, 198). These receptors do not possess intrinsic tyrosine kinase activity, but their activation leads to increased activity of soluble tyrosine kinases of the Src family (221). The activation of PLD in the RBL-2H3 mast cell line is abolished by tyrphostin and genistein, implying the involvement of these tyrosine kinases (108, 124). There is also a report of PLD activation in cells transformed by v-Src (196), although later work has shown a dependence on Ras (85) and the related small G protein Ral (85).

A role for a tyrosine kinase(s) in the activation of PLD by G protein-mediated agonists is also suggested by many studies. Dubyak et al. (40) and Kusner et al. (113) observed that ATP markedly augmented the stimulation of PLD by GTPγS in permeabilized U937 promonocytic leukocytes and that the effect was mimicked by vanadate, an inhibitor of protein tyrosine phosphatases, and blocked by tyrosine kinase inhibitors. These inhibitors have also been found to inhibit PLD activation by thrombin in platelets (137), by f-Met-Leu-Phe in neutrophils (211), by endothelin in vascular smooth muscle cells (222), by bombesin in fibroblasts (19), and by carbachol in HEK cells transfected with M3 muscarinic receptors (189). However, in most of these studies with intact cells, high doses of inhibitors were employed, and their specificity of action was not established. In one study that used many inhibitors (137), the inhibition of PLD did not correlate well with that of tyrosine phosphorylation.

However, there is additional evidence for the involvement of tyrosine kinase(s) in the effects of certain G protein-linked agonists on PLD. For example, in permeabilized HL-60 cells, Bourgoin and Grinstein (16) found a strong correlation between the effects of vanadyl hydroperoxide on tyrosine phosphorylation and PLD activity. Vanadate in combination with H_2O_2 also activated PLD in neutrophils (211) and platelets (137), and stimulatory effects of vanadate alone have been reported (189, 220). Because the cloning and expression of a regulated PLD has only just been reported (62), it is not known whether this or another isozyme is subject to direct phosphorylation and activation by a receptor-associated or soluble tyrosine kinase. Thus the role of such kinases in the direct regulation of PLD is presently unclear.

As described in section VII, there is evidence that tyrosine kinase activity is involved in upstream and downstream signaling involving Rho (154, 178, 181, 190). However, the specific involvement of tyrosine kinase(s) in Rho-mediated activation of PLD has not been defined. There is now much evidence that protein serine/threonine kinases are also targets of Rho family proteins (5, 103, 111, 135, 229). These have been designated p21-activated protein kinases and are related to the STE20 kinase that is involved in pheromone action in *S. cerevisiae* (135). Recently, a serine/threonine kinase that specifically binds to GTP-RhoA has been identified (122). Whether or not any of these serine/threonine kinases is involved in PLD regulation is presently unknown.

X. FUNCTIONAL SIGNIFICANCE OF PHOSPHOLIPASE D

Despite its ubiquitous distribution and regulation by a wide variety of extracellular signals, the physiological role of PLD remains unclear. Of the immediate products of its action, only PA seems to be important. The resting level of free choline in most cells is in the 0.1–0.3 mM range (163a), and this is deemed too high to fulfill a signaling role. However, chemical measurements of the intracellular concentration of choline and of radioactive choline in cells prelabeled with [^{3}H]choline indicate that it increases significantly during agonist stimulation, and there is usually increased release into the incubation medium (31, 37, 46, 116, 131, 136, 137, 170, 212). These results are, of course, consistent with PC being the major substrate of PLD in most cells. Lee et al. (119) have proposed that choline released as a result of constitutively active PLD in neuronal cells is utilized for acetylcholine synthesis.

Chemical measurements and studies in cells previously incubated with isotopic myristate or other fatty acids to selectively label PC indicate that PA increases rapidly during agonist stimulation. The magnitude of the final increase is comparable to that of DAG (46, 47). In contrast to the time course of DAG accumulation, which is often biphasic, PA formation is usually monophasic. The two phases of DAG are due to its initial formation from PIP_2 via PI-PLC, and then from PC via PLD and PAP (47).

Although there is evidence that PA per se is a signaling molecule, its immediate products DAG and LPA also have very important physiological functions. As alluded

to in section I, much of the DAG that accumulates in stimulated cells is derived from PC. In comparison with PC, which is the major phospholipid of all cell membranes, PIP_2 is present at a very low concentration (<0.1% of total phospholipid) and is rapidly exhausted, unless resynthesized, during agonist stimulation (4). Furthermore, the magnitude of DAG accumulation far exceeds the cell content of PIP_2 (4), indicating its origin from another source, with PC being the most likely source, based on measurements of choline production.

The major cellular function of DAG is to activate PKC isozymes. Initial reports suggested that DAG derived from PC was unable to activate PKC, as assessed by its translocation to membranes in stimulated cells (100, 135). However, further work revealed that the translocation was isozyme specific and that the first peak of DAG derived from PIP_2 breakdown elicited by thrombin in IIC9 fibroblasts caused only a transient translocation of PKC-α, which is a Ca^{2+}-dependent isozyme, whereas the second peak derived from PC breakdown was associated with sustained membrane association of PKC-ϵ, which is a Ca^{2+}-independent isozyme (58). On the other hand, the prolonged accumulation of DAG derived from PC breakdown induced by PDGF caused prolonged membrane association of PKC-ϵ, but not of PKC-α. Studies with a Ca^{2+} ionophore, Ca^{2+} chelators, and synthetic DAG indicated that PKC-ϵ could be translocated by an increase in DAG alone, whereas PKC-α required increases in both DAG and Ca^{2+} (58). Protein kinase C-δ was not detected in the IIC9 cells but would be expected to behave like PKC-ϵ. In accord with this, membrane translocation of PKC-δ and PKC-ϵ, but not of PKC-α, was observed in Swiss 3T3 cells incubated with PDGF, bombesin, and synthetic DAG (158). There has also been a report of differential translocation of PKC-α and PKC-ϵ in fibroblasts from Zellweger patients (whose cells have low levels of ether phospholipids) stimulated by bradykinin. The translocation of PKC-α was transient, whereas that of PKC-ϵ was sustained, and treatment of the cells with 1-*O*-hexadecylglycerol to enrich the ether-linked phospholipids blocked the membrane association of PKC-α, but not PKC-ϵ (26a). Because this treatment also blocked PLD activation, the results support a role for PKC-α, but not PKC-ϵ, in the regulation of PLD by bradykinin.

Although most studies have examined the translocation of PKC isozymes from cytosol to a crude membrane fraction, there have been some investigations of the intracellular localization of these isozymes and of their specific translocation to the plasma membrane, nucleus, and Golgi. The most complete study of subcellular localization utilized NIH 3T3 fibroblasts overexpressing various PKC isoforms (55). Immunofluorescence was utilized to reveal that the majority of the isozymes were not membrane bound but were diffusely distributed in the cytosol. With PMA treatment, the α- and ϵ-isozymes rapidly concentrated at the cell margins, and there was also association of PKC-α with the endoplasmic reticulum and of PKC-ϵ with the nucleus. Protein kinase C-βII associated with actin-rich microfilaments of the cytoskeleton, and PKC-γ accumulated in Golgi organelles. Thus activation of the cells with the phorbol ester caused a highly selective redistribution of PKC isozymes.

In a study of growing SH-SY5Y neuroblastoma cells, Leli et al. (121) found PKC-α and PKC-βI in the cytoplasm and plasma membrane, but not in the nucleus before or after PMA stimulation. Protein kinase C-ϵ was mostly present at the plasma membrane, but it was unclear whether or not it was associated with the nucleus. Some studies have specifically examined the translocation of PKC isozymes to the nucleus in stimulated cells. Leach et al. (118) first reported that thrombin stimulation of IIC9 cells caused an increase in the DAG content, PKC activity, and protein phosphorylation of subsequently isolated nuclei. However, in contrast to the above reports, Western blotting showed an increase in PKC-α but not PKC-ϵ. An immunofluorescence study by Khalil et al. (97) of aortic smooth muscle cells stimulated with the α_1-adrenergic agonist phenylephrine also failed to show translocation of PKC-ϵ to the nucleus, although it became associated with the plasma membrane.

In summary, the reports cited above and others reviewed by Buchner (22) indicate that the α-, γ-, δ-, ϵ-, and ζ-isozymes of PKC can be translocated to the nucleus in response to agonists in various tissues and cells. Thus agonist-induced PC breakdown would be expected to lead to increased phosphorylation of substrates in the nucleus and at the plasma membrane as a result of activation of PKC-δ and PKC-ϵ. Physiological substrates for PKC in or at the plasma membrane include myristoylated alanine-rich C-kinase substrate (MARCKS), which is present in most cells and is thought to be involved in chemotaxis and changes in cell shape and mobility (11), and pleckstrin, which becomes phosphorylated during platelet activation. Other plasma membrane-associated substrates are the receptors for epidermal growth factor and other growth factors, α_1-adrenergic receptors, Na^+/H^+ antiporter isoforms, and several types of ion channel (47). Neurogranin and neuromodulin are also prominent PKC substrates in brain. In many cases, phosphorylation of these substrates by PKC alters their activity. There are also many PKC substrates in nuclei (8). Some of these have been identified, e.g., lamin B2, nucleolar protein B23, RNA polymerase II, DNA topoisomerase I, CREB (cAMP response element binding protein) myogenin, and p53 (tumor suppressor protein) (22). Because many of these proteins are involved in regulating transcription, their phosphorylation could provide the link between PKC activation and changes in RNA/protein synthesis.

The mechanisms that cause PKC isozymes to be translocated to the nucleus are not known, except that an

increase in nuclear DAG has been reported in fibroblasts stimulated with α-thrombin (83, 118). Phosphoinositide-PLC and PC-PLD have been identified in the nucleus (3, 6, 171), but it is not known how they are activated.

Although production of DAG for sustained activation of PKC seems to be a major physiological role for PLD, it may be questioned why this could not be subserved by a PC-PLC. However, this enzyme has not been characterized in mammalian tissues, and its existence in animal cells is uncertain. Some functions have been attributed to PA per se. Many of these are the result of in vitro observations and include stimulation of PKC, especially PKC-ζ (123, 152), and of other protein kinases (14, 98, 142, 167). Other reports of in vitro effects of PA include stimulation of a protein tyrosine phosphatase (208, 230), PI-PLC-γ (76, 87), PIP 5-kinase (148), and *n*-chimaerin an activator of Rac GAP (1). Inhibitory effects have also been noted, namely of Ras GAP (209) and the interaction of Rac with Rho-GDI (26). Phosphatidic acid binding to Raf-1 kinase has also been reported (54). Inhibition of adenylate cyclase was reported in earlier studies, but the effect may be exerted on G_i (47). The status of this and other early reports of the stimulation of Ca^{2+} influx and mobilization remains unclear because of the probable contamination of the PA preparations with LPA. The physiological significance of the in vitro effects listed above remains unclear because few of them have been examined in vivo, and it is difficult to selectively elevate PA in cells or to introduce it into cells without further metabolism to DAG and LPA.

There is good evidence that PA plays a role in the activation of NADPH oxidase and the resulting respiratory burst that occurs in neutrophils stimulated by chemotactic peptides (47). Thus O_2^- formation is better correlated with changes in the production of PA than of DAG, although the latter has an effect (7, 183). Synergism between PA and DAG has been observed in the activation of NADPH oxidase in a cell-free system from neutrophils (176). Interestingly, McPhail et al. (142) have obtained evidence that the action of PA is mediated by a protein kinase. This was shown by the effects of protein kinase inhibitors and ATP depletion. A predominant kinase substrate was p47*phox* a component of NADPH oxidase.

There have been many reports of a mitogenic action of PA (47), but these are complicated by the possibility that the PA used in many of the studies may have been contaminated by LPA. Because PA was added directly to the cells, any trace LPA could have interacted with its cell surface receptor and promoted mitogenesis (213). However, the possibility remains that PA exerted an effect independent of LPA in some studies, since it was equipotent with LPA (214). Whereas there is information about the mechanism by which LPA stimulates mitogenesis (see below), the molecular basis of the action of PA is unknown.

Phosphatidic acid has been proposed to mediate the effects of α-thrombin and PDGF on actin polymerization, as shown by stress fiber formation and elongation of IIC9 fibroblasts (59). This is because direct addition of PA and PLD from *Streptomyces chromofuscus* to the cells reproduced the morphological effects of the agonists. The effects were not mimicked by other phospholipids or by PMA, synthetic DAG, or PLC from *Bacillus cereus*, indicating that they were not mediated by DAG formation or PKC activation. As presented in section VII, Rho has been strongly implicated in agonist-induced stress fiber formation and in the regulation of PLD. However, a role for PLD in agonist-induced actin polymerization is still speculative (146), and other mechanisms are possible.

The main metabolic fate of PA generated by PLD in cells is conversion to DAG, presumably by the plasma membrane-associated PAP isozyme (36, 82). Another fate is hydrolysis to LPA by a specific phospholipase A_2 (205). This reaction is prominent in platelets (43, 145), but probably occurs in other cells. However, it must be recognized that the role of PLD-derived PA in the generation of LPA is largely conjectural, and it may be formed by other mechanisms.

Lysophosphatidic acid is now recognized as an important intercellular messenger (79, 145–147). Its effects can be attributed to its interaction with a cell surface receptor (204) linked to G proteins of the G_q and G_i families (145). A 40-kDa protein has been identified by cross-linking to LPA (215), but it is unclear that this is the receptor. Prominent cellular effects of LPA include hydrolysis of PIP_2 leading to Ca^{2+} mobilization and PKC activation (78, 145, 146) and activation of PLD by a PKC-dependent mechanism (60, 145, 146, 216). There have also been reports of a decrease in cAMP (145), presumably mediated by G_i, and liberation of arachidonic acid due to activation of phospholipase A_2 (49, 188).

An important effect of LPA is stimulation of growth (81), and much of the growth-stimulatory action of serum appears to be due to LPA. The mitogenic action of LPA is inhibited by pertussis toxin, indicating that it probably involves G_i, and there is much evidence that it involves the Ras/MAP kinase signaling pathway (69, 71, 79, 110, 141, 145). The probable mediator of Ras activation is the βγ-subunit of G_i, and a nonreceptor tyrosine kinase may be involved (145, 146). Lysophosphatidic acid also activates PI 3-kinase, and this may be mediated by the βγ-subunit also (204, 228) or may be secondary to Rho activation (109).

Like other G_q- and G_i-linked agonists, LPA induces tyrosine phosphorylation of cellular proteins, including focal adhesion kinase and paxillin (69, 110, 141, 145, 146, 191). This is accompanied by recruitment of these and other proteins to focal adhesion sites in the cell and the initiation of focal adhesions and the assembly of actin stress fibers (145, 146). Rho is a critical component of the

tyrosine phosphorylation induced by LPA and other G protein-linked agonists, since treatment of cells with the *C. botulinum* C3 exozyme blocks the tyrosine phosphorylation of focal adhesion kinase and paxillin induced by these agents (145, 146, 178).

As a result of its effects on PIP_2 hydrolysis and the Ras/MAP kinase and Rho pathways, LPA produces dramatic effects on morphology, motility, and ion fluxes in a variety of cells. In NIE-115 and NG-108-15 neuronal cells, it causes rounding up, growth cone collapse, and neurite retraction (77, 81), and in PC-12 cells, it causes neurite retraction and dopamine release (192, 202). There is also activation of a Ca^{2+}-dependent Cl^- current in *Xenopus laevis* oocytes (41, 48) and induction of chemotaxis in *Dictyostelium discoideum* (80).

Other interesting effects of LPA include inhibition of gap-junctional communication in the WBF344 epithelial cell line (68). The effect is associated with activation of MAP kinase and phosphorylation of connexin-43. Lysophosphatidic acid also mimics the effect of serum to permit invasion of endothelial cells by an invasive clone (MM1) of rat ascites hepatoma cells (74). In embryonic development, LPA has been shown to increase the blastocyst ratio from two-cell to four-cell embryos (105). The effect was inhibited by pertussis toxin, implying the involvement of G_i or G_o.

In the foregoing description of the physiological role of PLD, emphasis has been placed on the actions of the products of PLD action. However, it is possible that the alterations in lipids caused by the enzyme might per se cause perturbations in the physical properties of membranes. Thus a localized change in the PC/PA of a membrane could affect membrane fusion and the binding or release of membrane-associated proteins. Studies with isolated rat liver plasma membranes incubated with GTPγS for 10 min showed a decrease in PC of ~13 nmol/mg protein and a release of choline of ~16 nmol/mg protein (12). Because the initial level of PC was 146 nmol/mg protein, these data suggest a breakdown of PC of ~10%. Studies by Higgins and Evans (66) have indicated that only 15% of the PC in rat liver plasma membranes is in the inner leaflet. Accordingly, if this were a physiological site of action of PLD, as seems likely, it can be surmised that agonists could induce major changes in its phospholipid composition. Similar reasoning could be applied to other membranes, in particular Golgi membranes which contain 5–15% of their total PC in the inner leaflet (65) and possess an ARF-responsive PLD (107, 171).

ADP-ribosylation factor is involved in vesicular transport in the Golgi complex and has been implicated in the fusion of microsomal vesicles, endosomes, and nuclear vesicles and in the formation of clathrin-coated vesicles (89, 150). In the Golgi, ARF is required for the binding of β-COP, a component of coatomers which are large protein complexes that cover the vesicles that traverse from the endoplasmic reticulum to the Golgi (150, 184). However, ARF may have additional functions in the membrane bilayer fusion that is involved in vesicle budding and binding, and it has been speculated that PLD may play a role in these (89, 150). This is supported by the finding that ethanol inhibits intra-Golgi vesicular transport (165) and receptor-stimulated secretion (199). Because ethanol inhibits PLD-mediated PA formation, due to the transphosphatidylation reaction, these findings suggest a role for PA in vesicular transport, perhaps because of its activity as a membrane fusogen (42, 150). There may be additional roles for PA and also PIP_2, which may be formed due to the activation of PIP 5-kinase (128, 150). The possibility that PLD might play a role in vesicle transport in Golgi could be extended to other vesicular membrane trafficking systems involved in both exocytic and endocytic pathways.

XI. CONCLUSION

With the cloning of the first PLD isozyme (62) and the probability that other isozymes will soon be cloned, information concerning the structure-function relationships and regulation of these enzymes should rapidly follow. Thus the catalytic domain(s) of the enzymes should be defined and the sites and mechanisms of interaction with PKC, ARF, Rho, PIP_2, oleate, and other potential regulators should be identified. In addition, molecular biological and immunological approaches should help greatly in the definition of the functions and the tissue and subcellular distribution of the various isozymes.

Information is now beginning to appear concerning the mechanisms by which growth factors, G protein-linked agonists, and other extracellular messengers regulate PLD, with emphasis on the roles of Rho and ARF. A critical issue is to define the mechanisms by which these small G proteins are activated by growth factors and other agonists. Another point of interest will be to discover what role PLD plays in the presently known cellular changes induced by activation of Rho and ARF. In short, the next few years should yield exciting developments concerning the cellular role of PLD.

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