The EMBO Journal Vol.18 No.22 pp.6339–6348, 1999

Loss of receptor regulation by a phospholipase D1 mutant unresponsive to protein kinase C

Yue Zhang[1], Yelena M.Altshuller[2], Scott M.Hammond[2], Andrew J.Morris[1,2,3] and Michael A.Frohman[1,2,3,4]

[1]Molecular and Cellular Biology Program, [2]Department of Pharmacological Sciences and [3]Institute for Cell and Developmental Biology, SUNY at Stony Brook, Stony Brook, NY 11794-8651, USA

[4]Corresponding author
e-mail: michael@pharm.sunysb.edu

Activation of phosphatidylcholine-specific phospholipase D (PLD) constitutes an important part of the cellular response to agonist signaling. PLD1 is stimulated *in vitro* in a direct and synergistic manner by protein kinase C (PKC), ADP-ribosylation factor (ARF) and Rho family members. However, the direct and specific role of each of these effectors in agonist-stimulated PLD activation is poorly understood. We have used transposon mutagenesis to generate a library of PLD1 alleles containing random pentapeptide insertions. Forty-five alleles were characterized to identify functionally important regions. Use of an allele unresponsive to PKC, but otherwise seemingly normal, to examine coupling of PLD1 to a subset of G-protein-coupled receptors demonstrates for the first time direct stimulation of PLD1 *in vivo* by PKC and reveals that this direct stimulation is unexpectedly critical for PLD1 activation.

Keywords: G-protein-coupled receptors/pentapeptide mutagenesis/phospholipase D/protein kinase C/RhoA

Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline (reviewed in Frohman *et al*., 1999). PLD activity and the production of PA have been implicated in signal transduction, membrane vesicular trafficking, cytoskeleton reorganization and cell proliferation (reviewed in Ktistakis, 1998; Jones *et al*., 1999). PLD activity is present in many mammalian cells and tissues and is upregulated in response to a wide variety of agonists that signal through heterotrimeric G-protein-coupled or tyrosine kinase receptors. Receptor stimulation initiates multiple signal transduction cascades, ultimately including activation of protein kinase C (PKC), ADP-ribosylation factor (ARF) and Rho family members. Since these effectors stimulate downstream effector pathways that ultimately regulate cellular morphology, proliferation and secretion, there has been intense interest in determining how stimulation of PLD fits into these cascades.

However, despite the consensus that PKC, ARF and Rho family members directly and potently stimulate PLD (specifically PLD1) *in vitro* (Hammond *et al*., 1997), the relative importance of each effector class in stimulation of PLD *in vivo*, and whether synergy between them is required, has remained unclear for several reasons. First, there are multiple types of mammalian PLD activities, including the cloned and related phosphatidylinositol 4,5-bisphosphate (PIP_2)-dependent, PC-specific PLD1 (Hammond *et al*., 1995) and PLD2 (Colley *et al*., 1997) enzymes. Secondly, activation of the effectors *in vivo* is inextricably intertwined; for example, direct stimulation of PKC by the diacylglycerol (DAG) analog phorbol ester, phorbol 12-myristate 13-acetate (PMA), can result in activation of Rho and ARF (Watanabe *et al*., 1997; Fensome *et al*., 1998), and inhibition of Rho activity can block PKC activation (Hippenstiel *et al*., 1998). Thirdly, the pharmacological inhibitors historically employed to inhibit each of these effector pathways are now appreciated to have complex effects on cellular responses. For example, inhibitors of PKC catalytic activity are quite potent at blocking PLD1 stimulation *in vivo* (e.g. Yeo and Exton, 1995; BoyanoAdanez *et al*., 1997; Min *et al*., 1998). However, PLD1 is stimulated through interaction with the regulatory domain of PKC, the stimulation does not involve kinase activity, and it is not blocked by these inhibitors *in vitro* (Hammond *et al*., 1997; S.M.Hammond and A.J.Morris, manuscript submitted); thus, the *in vivo* inhibition is clearly mediated through unknown indirect effects on cellular pathways (reviewed in Houle and Bourgoin, 1999). Inhibition of PKC activation through the regulatory domain inhibitor calphostin is also quite potent at blocking PLD stimulation (Min *et al*., 1998; Mamoon *et al*., 1999). However, calphostin is now appreciated to be an extremely potent direct inhibitor of PLD1 and PLD2 (S.M.Hammond and A.J.Morris, manuscript submitted). Rho inhibitors (bacterial toxins) decrease the levels of PIP_2 (Schmidt *et al*., 1996; Rumenapp *et al*., 1998), which is required as a cofactor for PLD stimulation by any effector. Finally, PLD inhibition by the ARF inhibitor Brefeldin A is achieved only at concentrations that cause profound effects on cellular organization (Meacci *et al*., 1999).

We have been approaching these issues by attempting to develop PLD mutants selectively unresponsive to individual effectors (Sung *et al*., 1997, 1999a,b). The molecular cloning of human PLD1 revealed that it exhibited a low basal activity and was stimulated synergistically by conventional PKC (PKCα and -β) and members of the ARF (ARF1-6) and Rho (RhoA, Cdc42 and Rac) families *in vitro* (Hammond *et al*., 1995, 1997). This led to the hypothesis that PLD1 would be involved in one or more of the downstream cell biological pathways associated with each of these classes of effectors. Furthermore, the synergistic activation of PLD1 by the different classes of effectors suggested that they would be found to interact with and stimulate PLD1 through distinct domains, as has

since been confirmed (Sung *et al*., 1997, 1999b; Yamazaki *et al*., 1999). Deletion of different regions has revealed that the PLD1 N-terminus (amino acids 1–325) is required *in vitro* for stimulation by PKCα, but not for stimulation by ARF and Rho (Sung *et al*., 1999b). However, this deletion also removes domains thought to be important for normal functioning *in vivo* and results in a loss of membrane association, making it unattractive as a tool for investigating signaling pathways. RhoA has been shown to interact with PLD1 within the C-terminal 362 amino acids, a large region containing numerous sites required for catalytic activity (Sung *et al*., 1997; Yamazaki *et al*., 1999). Finally, the site conferring ARF stimulation is undefined, although the N-terminus and loop region have been ruled out as possibilities (Sung *et al*., 1999b).

In this report, we describe a large-scale random mutagenesis approach to scan PLD1 for sites required for its different functional properties and to identify mutants useful for cell biology studies. We report an allele normal in all respects examined save for a complete lack of response to PKC. Analysis of the behavior of this allele in some well established G-protein-coupled receptor signaling pathways suggests that PKC stimulates PLD1 directly, and that in order to activate PLD1 significantly *in vivo*, this direct stimulation is required. Taken together with reports on the role of Rho (Schmidt *et al*., 1996), our results suggest that signal integration through both PKC and small G-proteins is required for physiological *in vivo* activation of PLD1 during agonist signaling.

Results

Generation of a library of randomly mutated PLD1 alleles using a modified pentapeptide insertion strategy

Pentapeptide insertion mutagenesis (PIM) is a method using transposon insertion and almost reciprocal excision through which a five amino acid peptide is introduced randomly into target proteins to induce local changes in structure (Hayes *et al*., 1997). PIM offers several advantages over other mutagenesis methods, including each mutant containing precisely one insertion that is easily located using restriction analysis. In contrast, the frequency of mutations obtained using PCR-based methods is difficult to predict and quite variable over the length of a target, and sequencing the entire target sequence is required.

In our initial experiments >90% of the transposon integration events occurred in the plasmid backbone. Accordingly, the protocol was modified to recover only the plasmids targeted in the cDNA insert (Figure 1). Of 111 individually targeted pCGN-hPLD1b plasmids generated using the modified method, all but three were targeted correctly. The remaining 108 insertions were located at 69 individual sites, some of which were represented frequently (putative 'hot-spots' for mutagenesis by this method). Of the 69 unique integration events, five were in the 3′ UTR sequence and eight resulted in the generation of a stop codon (Figure 2).

Identification of regions required for PLD1 activation, membrane localization and protein modification using PIM analysis

A set of 45 well distributed alleles were chosen, with the longest region left uninterrupted 83 amino acids in length.

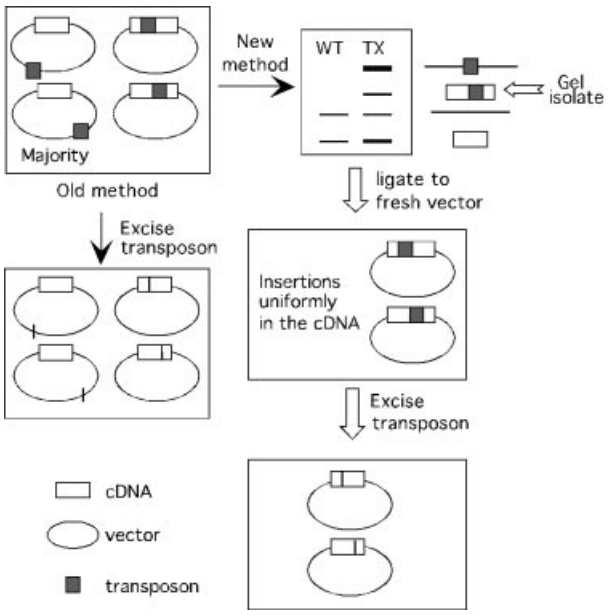


Fig. 1. A modified pentapeptide insertional mutagenesis strategy. The classic PIM scheme (Hayes *et al*., 1997) involves insertion of a 4000 bp bacterial transposon into the target plasmid at random sites (top left), and then excision using *Kpn*I, which is present at each end of the transposon insertion (middle left). Excision results in the persistence of a 15 nt sequence that consists of GGGGTACCCC followed by 5 nt duplicated from the insertion site of the host plasmid. Regardless of the reading frame, the insertion will encode a glycine (GGx) and a proline (CCx) residue. When the insertion occurs after the second nucleotide in a tyrosine codon, it generates a stop (TAG) codon. As modified, a cloning step is added, in which cDNAs containing transposon insertions are selected (top right) and ligated to fresh vector (middle right). This results in all of the transposons being located in the cDNA intended to be targeted.

The PLD1 PIM alleles were assayed for activity *in vitro* in response to ARF1, RhoA and PKCα to identify regions of the protein important for enzymatic activity and effector stimulation (Figure 3A). PLD1 is characterized by regions that are unique to PLD1 and by regions that are conserved among PLDs from multiple species (reviewed in Frohman *et al*., 1999). In three regions, specifically the unique N-terminus (amino acids 1–98) and loop regions (505–622), and the conserved PX domain (99–213), relatively modest or no consequences ensued from the insertions, with the exception of PIM116 and PIM200, both of which exhibited decreased activity, and PIM87, which exhibited a selective loss of response to PKCα. In all other regions, most insertions resulted in a parallel loss of responsiveness to ARF1, RhoA and PKCα, which presumably reflects altered functioning of the basic enzymatic process, since the effectors stimulate PLD1 at distinct sites in the protein (Hammond *et al*., 1997). Western blotting revealed that all alleles were expressed at easily detectable levels, ruling out protein instability as a cause for the lack of activity (Figure 3B and our unpublished data).

Two other PLD1 properties, subcellular localization and post-translational modification, were assessed in correlation with enzymatic activity. Wild-type PLD1 is membrane-associated and segregates on SDS–PAGE as a doublet as a result of an unknown post-translational modification (Figure 3B, WT pellet lane; Sung *et al*., 1997; Manifava *et al*., 1999). All alleles that separated as a doublet (as opposed to the appearance of the lower

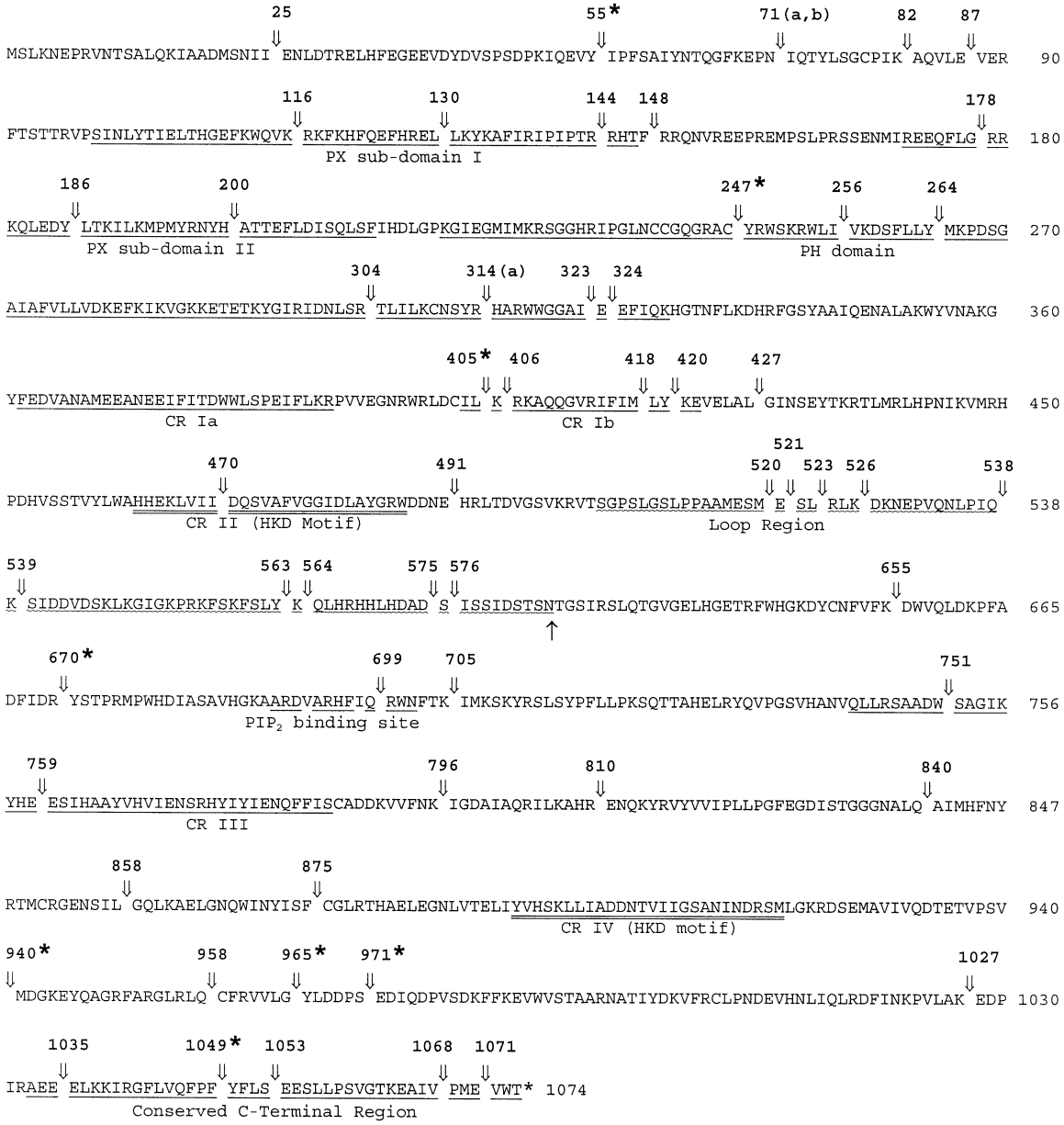


Fig. 2. Distribution of PLD1 PIMs. The protein sequence of PLD1b is shown, along with the sites at which insertions were found. PLD1b was used for this insertional mutagenesis study, but to maintain consistency with previous publications (Hammond *et al*., 1995, 1997), the numbering for PLD1a is used. PLD1a and PLD1b differ in that PLD1a contains an extra 38 amino acid exon inserted at N585 (arrow) but the isoforms are identical with respect to their regulation (Hammond *et al*., 1997) and subcellular localization (Toda *et al*., 1999). Key domains in PLD1 are underlined. The PIMs are indicated by the amino acid residue number after which the insertion occurs. Multiple insertions at the same site are indicated by 'a' or 'b'. * indicates a stop codon introduced by the insertion. CR, conserved region; PX, phox; PH, pleckstrin homology domain.

band only) were also predominantly membrane-associated (Figure 3B and our unpublished data). In contrast, the majority (e.g. PIM256, 699, but not all, e.g. PIM200) of the alleles that electrophoresed as a single band exhibited partial redistribution to the cytosol. Although appearance of the doublet/membrane localization correlated with PLD1 activity *in vitro* (Figure 3A), several alleles localized normally but were inactive (e.g. PIM705), and several active alleles exhibited redistribution and electrophoresed as a singlet (e.g. PIM810), similar to a previously described N-terminally truncated PLD1 mutant (Δ1–325) that is responsive to ARF1 and RhoA (Sung *et al*., 1999b). Thus, predominant membrane association and the post-translational modification that results in the migration as a doublet are not strictly required for enzymatic activity *in vitro*, nor do they necessarily signify active protein.

There were two major regions in which insertion of pentapeptides resulted in altered localization: amino acids 200–470 and 699–960. A PH domain and half of the catalytic site lie within the first region, and a motif that may interact with the PC substrate, the other half of the catalytic site and the PIP_2-interacting site (Sciorra *et al*., 1999) are in the second.

The assay presently in wide use for measuring PLD activity *in vitro* (Brown *et al*., 1993) was developed and optimized for wild-type PLD1 as a model for its regulated activity *in vivo*. To confirm that the *in vitro* activity observed for the PIM alleles reflected their behavior

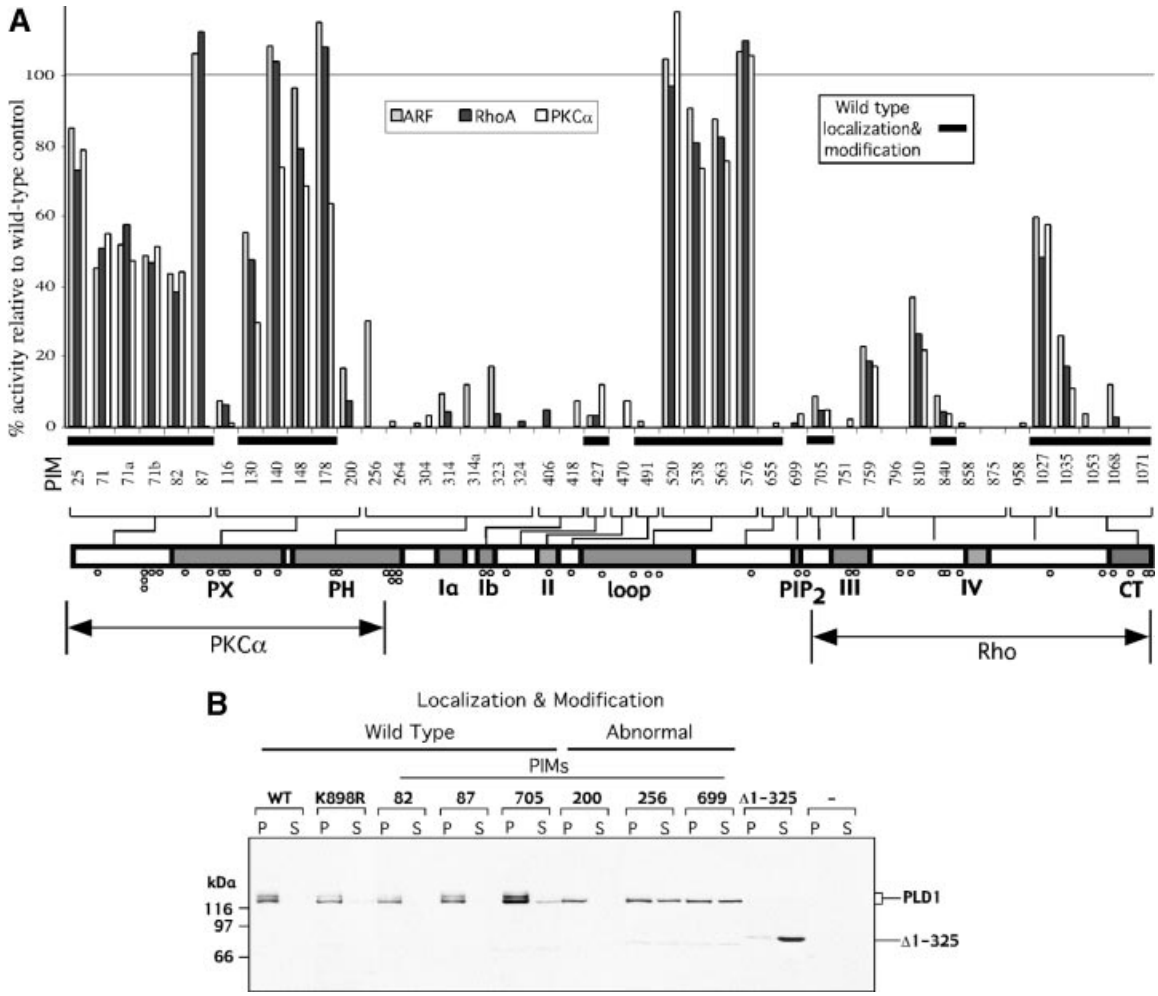


Fig. 3. (**A**) Effector activation and membrane association scanning analysis of the PLD1 PIMs. Lysates from each PIM overexpressed in COS-7 cells were stimulated with activated ARF1, RhoA and PKCα. Their PLD activities were assessed using the *in vitro* head-group release assay and then compared with the activities exhibited by wild-type PLD1 and PLD1-K898R, a catalytically inactive allele (Sung *et al*., 1997), which defined the 100 and 0% activities, respectively. The basal and stimulated activities observed for cells overexpressing PLD1-K898R were not significantly different from those observed for cells transfected with the expression plasmid for Gbx-2, an irrelevant protein, or the empty vector, as reported previously (Sung *et al*., 1997, 1999a,b). Each PIM was assayed in duplicate a minimum of two times. The variability between duplicates averaged 3%. The inter-assay variability was higher (~10%) due to small differences in transfection efficiency for individual plasmids in the separate experiments. Activities within 10% of the PLD1-K898R negative control were frequently not reproducibly significant above background. Membrane localization was assessed as described below. PIMs exhibiting wild-type membrane localization and modification are denoted by thick underlining. The relative position of each PIM is indicated by a small circle underneath the bar figure, which depicts boxes representing the conserved domains and unique features of PLD1. CT, C-terminus; Ia, Ib, II, III, IV, conserved regions; PIP_2, PIP_2 binding site. Underneath the bar figure are brackets indicating the regions previously reported to be involved in PKCα and Rho activation. (**B**) Western analysis of wild-type and mutant proteins after fractionations. COS-7 cell lysates were centrifuged at 30 000 *g* for 30 min to separate the membrane associated pellet (P) from the cytosolic supernatant (S). The recombinant proteins were detected using a monoclonal antibody (3F10) directed against the HA-epitope tag that was fused to the N-terminus of PLD1, as described previously (Hammond *et al*., 1995; Colley *et al*., 1997). PLD1 migrates as a doublet at ~120 kDa. WT, wild-type PLD1; '–', untransfected COS-7 cells. The data shown are representative of four experiments.

in vivo, we used as a simple test, PMA stimulation of cells to activate PKC, which then in turn stimulates PLD1. All of the PIM alleles when overexpressed in HEK293 cells exhibited responses *in vivo* to PMA over a 30 min time period that paralleled their responses *in vitro* to activated PKC (our unpublished data), except for PIM116, which was more active *in vivo* (~40% as active as wild-type PLD1).

PIM87 is non-responsive to PKCα and -β in vitro but otherwise similar to wild-type PLD1

PIM87 did not respond to PKCα *in vitro* when overexpressed and assayed in COS-7 cell lysates (Figure 3A). To characterize this further, we purified PIM87 and re-examined its response *in vitro* to each PLD effector (Figure 4). PIM87 was expressed in sf9 insect cells at levels similar to wild-type PLD1 and was membrane-associated (our unpublished data). Using the effectors at their maximally stimulating concentrations, we found that within the limits of accuracy (~5%) and sensitivity (~2%) of the assay, PIM87 and wild-type PLD1 respond similarly to RhoA and ARF1, but PIM87 is not stimulated by PKC-β (or PKCα, data not shown). Dose–response curves were generated using ARF1 and PKCα. ARF1 stimulated PIM87 and PLD1 with similar efficacies, whereas PKCα at >10-fold above the amount required to minimally stimulate wild-type PLD1 failed to activate PIM87 (data not shown). Finally, synergistic stimulation of PLD1 and

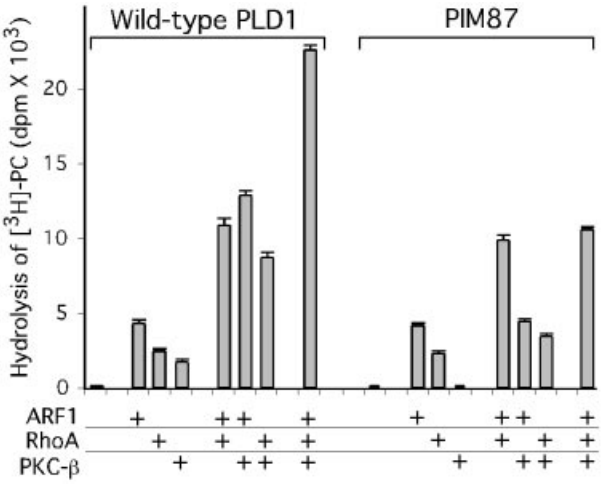


Fig. 4. PIM87 is selectively non-responsive to PKC-β. Wild-type PLD1 and PIM87 were expressed in sf9 cells using the baculoviral system and immunopurified as described previously (Hammond *et al*., 1997). PLD activation was determined using the head-group release assay in the presence of maximally effective concentrations of ARF1, RhoA and PKC-β alone or in combination. Samples were assayed in triplicate and a representative of three experiments is shown.

PIM87 by ARF1, RhoA and PKC-β was addressed. Whereas PKC-β synergized with ARF1 and RhoA to increase activity of wild-type PLD1 by 3- to 3.6-fold over ARF1 or RhoA alone (Figure 4, left panel), no such synergistic increase was observed for PIM87 (right panel). Synergistic stimulation of wild-type PLD1 and PIM87 by ARF and Rho was comparable (~3-fold), and an additional 2-fold increase was observed with the addition of PKC-β for wild-type PLD1, but not for PIM87. Thus, PKC does not stimulate PIM87 directly, or synergistically in combination with ARF and/or Rho.

PIM87 is non-responsive to PMA in vivo but otherwise similar to wild-type PLD1

The simplest prediction for the behavior of PIM87 in *vivo* would be to expect it to fail to respond to PMA stimulation, and this was observed after overexpression in HEK293 cells (Figure 5A). An alternative explanation would be that it is simply not active *in vivo*. However, this is not the case, as was found by examining the PIM87 response to RhoA: overexpression of wild-type PLD1 in unstimulated cells does not result in increased PLD activity in HEK293 cells compared with overexpression of the catalytically inactive PLD1-K898R (e.g. Figure 5A, 0 and 15 min basal samples, Figure 5B), reflecting the low intrinsic basal activity for the enzyme and a lack of detectable activation by any endogenous factor. In contrast, co-transfection of wild-type PLD1 and wild-type RhoA results in a 2- to 2.5-fold increase in PLD1 activity relative to co-transfection of PLD1-K898R and wild-type RhoA (Figure 5B). This presumably reflects an increase in cellular RhoA, some of which becomes GTP-loaded, membrane-associated and accordingly able to stimulate PLD1 to a modest extent. Although less dramatic than the response to PMA, the stimulation is significant, reproducible and consistent with prior reports (Park *et al*., 1997). A more dramatic stimulation is observed for co-expression of PLD1 and (dominant-active) $RhoA^{Val14}$, which results in a 4-fold stimulation of PLD activity. This reflects the fact that all of the

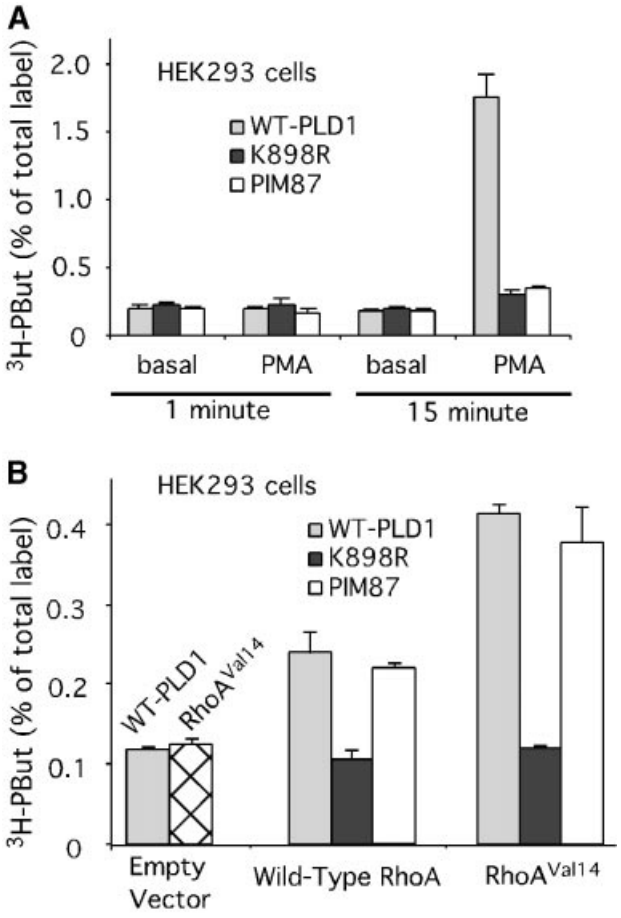


Fig. 5. PLD1-PIM87 does not respond to PMA but can be activated *in vivo* by RhoA. (**A**) HEK293 cells overexpressing wild-type PLD1, PLD1-K898R or PIM87 were stimulated with PMA for the times indicated and assayed for PLD (transphosphatidylation) activity *in vivo*. (**B**) HEK293 cells co-transfected with wild-type PLD1, PLD1-K898R or PIM87 and wild-type RhoA or dominant-active $RhoA^{Val14}$ were assayed *in vivo* for PLD activity for 30 min. The data shown are from one of three experiments performed in triplicate with similar results.

overexpressed Rho is in the active form and membrane-associated, leading to a greater stimulation of PLD1. Similar levels of activation are observed when wild-type or dominant-active RhoA are co-expressed with PIM87 (Figure 5B), leading to the conclusion that PIM87 is capable of being activated *in vivo* and it responds to stimulation by RhoA with similar efficiency to wild-type PLD1. It is likely that PIM87 also responds normally *in vivo* to ARF, although this is difficult to demonstrate since co-expression of PLD1 and dominant-active ARF does not result in PLD1 stimulation (Park *et al*., 1997).

PLD1 localizes to peri-nuclear vesicular structures (Colley *et al*., 1997; Toda *et al*., 1999). The subcellular localization of PIM87 in COS-7 cells as determined using immunohistochemistry was found to be indistinguishable from that of wild-type PLD1 (our unpublished data). PIM87 was also expressed at wild-type levels and electrophoresed as a doublet on SDS–PAGE similar to wild-type PLD1 (Figure 3B).

Taken together, these results demonstrate that PIM87 is unresponsive to PKC but wild-type in all other known respects and that it represents a unique tool with which

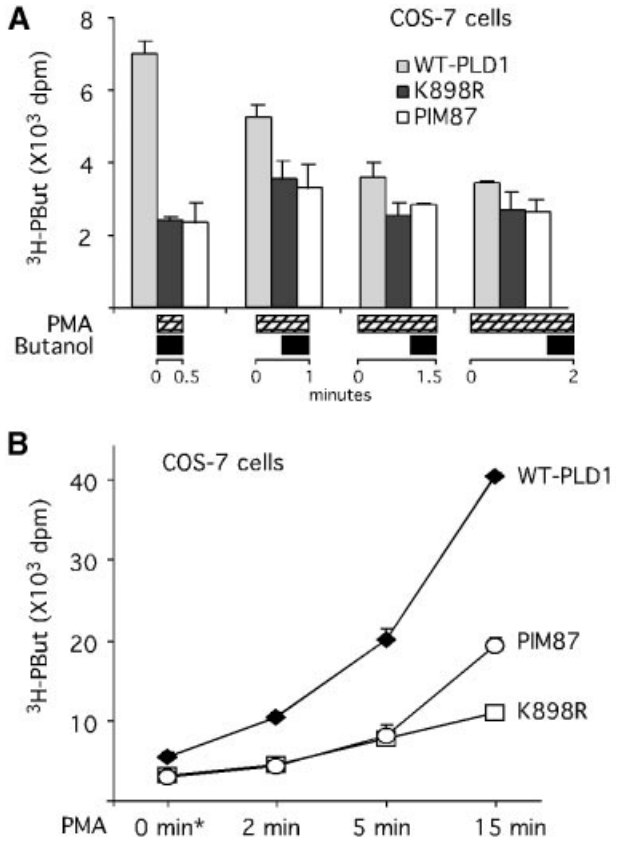


Fig. 6. PIM87 is modestly stimulated by PKC through an indirect pathway in COS-7 cells. (**A**) COS-7 cells overexpressing wild-type PLD1, PLD1-K898R or PIM87 were stimulated with PMA. Butanol was added at 30 s intervals to different dishes and lipids were extracted for TLC analysis 30 s after the addition of the butanol. (**B**) PMA and butanol were added at time 0 and lipids extracted at the times indicated for analysis. *PMA was not added to the 0 time point samples, but they were exposed to butanol for 15 min. An experiment performed in triplicate representative of four is shown.

the role of PKC in agonist-activated PLD signaling can be studied.

Indirect stimulation of PIM87 by PKC in COS-7 cells

The degree to which inhibitors of PKC catalytic activity block PLD stimulation varies with the agonist and cell line studied (BoyanoAdanez *et al*., 1997), suggesting that the indirect mechanism activated by PKC varies in potency in different settings (reviewed in Houle and Bourgoin, 1999). In preliminary experiments we found a small response in COS-7 cells for PIM87 to PMA (10–30% of the wild-type PLD1 response, our unpublished data), suggesting that the indirect pathway might be more easily observed in this cell line.

We explored this by examining in detail the response of PIM87 expressed in COS-7 cells to PMA. The temporal response of endogenous PLD to PMA or agonists in many cell lines is extremely rapid and levels of PLD activity are highest during the first few minutes of stimulation. Wild-type PLD1 overexpressed in COS-7 cells behaves similarly to the kinetics reported for endogenous PLD, in that the most dramatic level of activity is observed during the first 30 s (Figure 6A), although the enzyme continues to be active for at least an hour (Figure 6B and Colley *et al*., 1997). In contrast, PIM87 exhibited absolutely no elevation of activity over the inactive PLD1-K898R control during the first 2 min (Figure 6A) and responded only modestly at the 15 min time point (Figure 6B). This result indicates that there is a qualitative difference in the responses of wild-type PLD1 and PIM87, suggesting that the dramatic rapid activation characteristic of wild-type PLD in this and many other settings is due to the direct stimulation of PLD by PKC. One obvious route for the delayed response of PIM87 would be through ARF or Rho secondarily activated by PMA stimulation (Watanabe *et al*., 1997; Fensome *et al*., 1998).

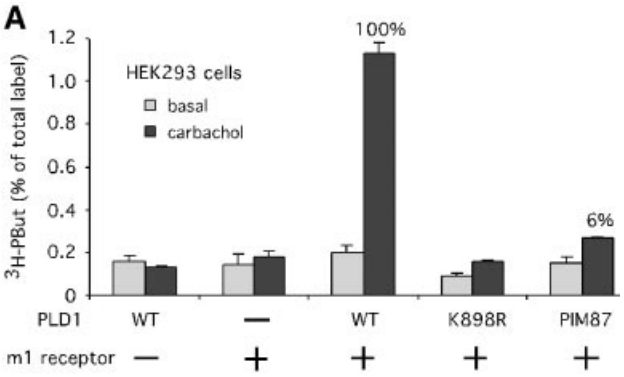
Dissection of G-protein-coupled receptor signaling in HEK293 cells using PIM87

Having established that PIM87 appears to behave normally *in vivo* except for its lack of direct response to PKC, we next examined the requirement for direct stimulation of PLD1 by PKC during agonist stimulation through G-protein-coupled receptors. Carbachol stimulation of the m1 and m3 muscarinic receptors results in immediate activation and translocation of PKC, Rho and ARF (Rumenapp *et al*., 1995; Keller *et al*., 1997), and stimulation of endogenous PLD (BoyanoAdanez *et al*., 1997). m1 muscarinic receptor stimulation of PLD has been reported to be abrogated by inhibitors of PKC (BoyanoAdanez *et al*., 1997; Mamoon *et al*., 1999), although as discussed above, these experiments do not address the direct stimulation of PLD1 by PKC. Moreover, one of the most widely used PKC catalytic inhibitors, bisindolylmaleimide, actually binds directly to muscarinic receptors and inhibits them (Lazareno *et al*., 1998).

Carbachol stimulation of HEK293 cells co-transfected with wild-type PLD1 and the m1 muscarinic receptor resulted in a 6-fold increase in PLD activity *in vivo* above that observed for PLD1 or the m1 receptor alone, or the m1 receptor co-transfected with PLD1-K898R (Figure 7A). PIM87 is stimulated in this setting only minimally (6%), barely reaching levels of significance in the assay. This result suggests that virtually no PLD1 stimulation is observed unless there is a direct stimulation by PKC, even though Rho and ARF are activated in this setting. This type of analysis was also performed to examine m3 muscarinic and bombesin receptor signaling to PLD1 (Figure 7B). The bombesin receptor has been reported to stimulate PLD both through PKC-dependent and PKC-independent pathways (Briscoe *et al*., 1995; Hou *et al*., 1998) with the usual caveats, whereas a critical role for Rho has been documented for the m3 muscarinic receptor (Schmidt *et al*., 1996, 1998). The tabulated and summarized results for the muscarinic and bombesin receptors were strikingly similar (Figure 7B); significant but only very small responses were observed for PIM87, suggesting that direct stimulation of PLD1 by PKC plays a vital role in PLD1 activation via G-protein-coupled receptor signaling.

Synergism between Rho and PKC pathways in PLD stimulation

Our finding that direct PKC stimulation appears to be critical for effective PLD1 activation via the m3 muscarinic receptor was not necessarily contradictory to prior reports concerning Rho in this setting, since it is possible that



B

	% of wild-type
m1 muscarinic receptor	5±1%
m3 muscarinic receptor	9±7%
bombesin receptor	11±5%

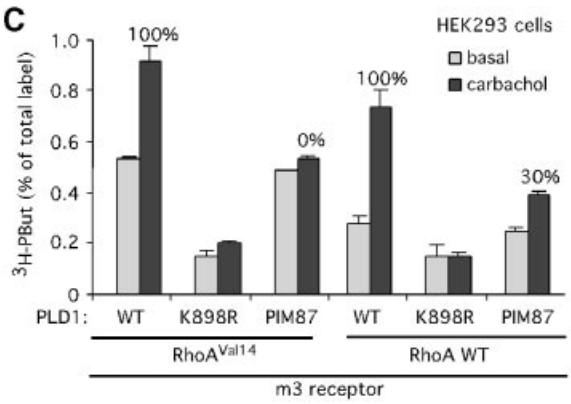


Fig. 7. PLD activation by G-protein-coupled receptors required direct stimulation by PKCα. (**A**) HEK293 cells co-transfected with the m1 muscarinic receptor and PLD1 alleles as indicated were stimulated with 100 μM carbachol for 30 min. PLD *in vivo* assays were performed in triplicate. The data shown are representative of two similar experiments. The percentage activation of PIM87 was calculated by subtracting the basal activity of PIM87 and the agonist-dependent increase of K898R (which represents the activation of endogenous PLD) from the agonist-activated PIM87 sample, and comparing this value with the agonist-activated wild-type PLD1 sample treated similarly. (**B**) Average agonist-stimulated activation of PIM87 when co-transfected with the m1 muscarinic, m3 muscarinic or bombesin receptors. The experiments were conducted in triplicate and the values shown represent the average of three experiments. (**C**) Triple transfection of PLD1 alleles, RhoA alleles and the m3 muscarinic receptor. One experiment representative of three is shown. Western blot analysis of control transfection dishes was used to confirm that the wild-type and mutant Rho and PLD proteins were expressed to similar levels (data not shown).

Rho and PKC synergize *in vivo* as well as *in vitro* and thus are both required for effective stimulation *in vivo*. To explore this issue, we carried out triple-transfections in HEK293 cells using the m3 muscarinic receptor, wild-type or constitutively active RhoA and the PLD1 wild-type or mutant alleles. As shown earlier in the right panel of Figure 5B, and now in the left panel of Figure 7C (light gray bars), transfection of wild-type PLD1 and PIM87 with RhoA^{Val14} leads to increased PLD1 activity in the absence of agonist stimulation. With agonist stimulation (dark gray bars), a very small increase in activity potentially due to endogenous PLD stimulation is observed in cells transfected with PLD1-K898R, an increase of comparable magnitude is observed for PIM87, and a much larger increase is observed for wild-type PLD1. These results demonstrate that when constitutively active RhoA is present in the cells in excess, no further activation of PIM87 (or, presumably, wild-type PLD1) can be achieved through agonist-stimulated Rho pathways. The results therefore suggest that the increase in activity observed for wild-type PLD1 is accomplished through direct stimulation by activated PKC.

In contrast, when the experiment is carried out using wild-type Rho (Figure 7C, right panel), an agonist-specific response for PIM87 is observed, although it is smaller than that observed for wild-type PLD1. We interpret this to suggest that when wild-type RhoA is present in the cell in excess, further activation of PIM87 and wild-type PLD1 can be achieved via Rho-stimulated pathways, through the receptor-driven activation and recruitment of RhoA to membranes. A much larger increase is seen though when the PLD1 is capable of responding both to RhoA and PKC. These results, taken together with prior work on the role of Rho in this setting, indicate that response of PLD1 to G-protein-coupled receptor signaling as explored here requires participation of both RhoA and PKC to achieve maximal levels of activation.

Discussion

A PLD1 pentapeptide insertional mutant library

In this report, we describe the use of a modified transposon pentapeptide mutagenesis scheme to generate a library of alleles of human PLD1b. The set of alleles and C-terminally deleted protein fragments generated should be useful for mapping regions of PLD1 involved in modifications such as phosphorylation (reviewed in Houle and Bourgoin, 1999) and palmitoylation (Manifava *et al*., 1999), or interactions such as with RhoA (Sung *et al*., 1997; Yamazaki *et al*., 1999), PKCα (Sung *et al*., 1999b) and inhibitors of PLD1 (Lee *et al*., 1997). The mutational approach successfully uncovered an allele that is selectively non-responsive to PKCα and -β but did not identify alleles selectively unresponsive to ARF or Rho. This may signify that the ARF and Rho binding sites are located close to regions required for catalytic activity. Little is known about the ARF-interacting site, except that it is not in the N-terminus or in the loop (Sung *et al*., 1999b). The Rho-interacting site is in the C-terminus as defined by two-hybrid analysis (Sung *et al*., 1997; Yamazaki *et al*., 1999), and C-terminal fragments of several of the C-terminal PIMs fail to interact with RhoA^{Val14} in this assay (Y.M.Altshuller, M.Yamazaki, Y.Zhang, Y.Kanaho and M.A.Frohman, manuscript in preparation), but all of these PIMs are largely or completely enzymatically inactive. It is most likely that other, more subtle methods will be required to generate RhoA- or ARF-selectively non-responsive alleles.

Regions of PLD1 involved in enzymatic activity and membrane association

We had previously reported a number of findings based on deletion analysis of PLD1 (Sung *et al*., 1999b). First,

the N-terminus (amino acids 1–325, which contain the PX and PH domains) was not required for enzymatic activity, because PLD1(Δ1–325) could be stimulated *in vitro* by RhoA and ARF1 (although not by PKCα). Secondly, the loop region was dispensable because it could be removed with only minor changes to the known properties of the enzyme. Thirdly, a free C-terminus was critical, since appending epitope tags to it or making small truncations rendered PLD1 inactive.

The PIM mutant analysis extends these results. First, the region containing the first 200 amino acids, which includes the PX domain, is not important for the properties we assayed, with the exception of the site targeted by PIM87 that results in a PKC-non-responsive allele. The N-terminal region, particularly the PX domain, may play a role *in vivo* in interaction with membrane vesicle proteins (reviewed in Frohman *et al*., 1999) or some other behavior that we have not yet examined. Secondly, not only is the loop region not important, but its internal structure is not harmful when disrupted, suggesting that this region of the protein is spatially dissociated from the catalytic, membrane- and effector-interacting sites. The loop region may also play some important role *in vivo* that has not yet been identified, although little evidence presently exists to support this. Finally, the C-terminus was again found to be highly sensitive to modification. Even pentapeptide insertions at three or six amino acids from the C-terminal end of the protein (PIM1068 and 1071) resulted in inactive protein, compared with less distal PIMs (e.g. PIM1027).

We had previously proposed that the C-terminus was not involved in membrane localization (Sung *et al*., 1999a), and the PIM findings support this. PIMs 1027–1071 were membrane-localized and post-translationally modified similarly to wild-type PLD1, even though they were not enzymatically active, suggesting that the function of this region is involved in promoting catalysis or effector stimulation. Since this region is conserved (Sung *et al*., 1999a) in mammalian PLD1 and PLD2, both of which can be activated by effectors, but not in yeast PLD, which is PIP_2-dependent but otherwise constitutively active (Rose *et al*., 1995), we presently prefer the hypothesis that it is involved in effector stimulation.

In contrast to the C-terminal PIMs, two regions of the protein (amino acids 200–470 and 699–960) contained a large number of PIMs that were inactive and exhibited partial redistribution from the membrane/cytoskeleton to the cytosol. Similar partial redistribution was reported previously for the inactive PLD1-S911A allele, which affects palmitoylation (Manifava *et al*., 1999). Taken together, these results suggest as one possibility that PLD1 subcellular localization is complex, and only part of it (e.g. membrane-associated but not cytoskeletal- or receptor-associated PLD1) is affected by the PIM mutations. The regions that have an effect include the PH domain, region I, both parts of the split-catalytic site (regions II and IV), the PIP_2-interaction site and region III (which has been proposed to interact with the choline head-group of PC; Sung *et al*., 1997). All of these regions are likely to interact with the membrane surface, and our results suggest that disruption of any of the regions interferes with overall membrane association and activity. Two points are worth noting: first, the normal localization and the PH domain are not required for activity, since the PLD1(Δ1–325) deletion mutant is active (Sung *et al*., 1999b); and secondly, interaction with PIP_2 is not required for membrane association of PLD2 (Sciorra *et al*., 1999), but redistribution is observed for PIM699, which inserted into the region that interacts with PIP_2. Thus, our results suggest that misalignment of any of the membrane interacting regions may result in aberrant interaction of the entire protein with the membrane surface and subsequently the catalytic site to fail to engage its membrane lipid substrate correctly (e.g. at an acceptable orientation and depth relative to the membrane surface).

Generation of a PKC-non-responsive PLD1 allele

We report here the identification of a selectively activated PLD1 useful for *in vivo* studies. The previous PKC-non-responsive allele reported, PLD1(Δ1–325), was cytosolic in location (Figure 3B) and seemingly inactive *in vivo*, which made it unattractive for *in vivo* experimentation (Sung *et al*., 1999b). PIM87 is normal in every respect examined except for its lack of response to direct PKC stimulation. It should be noted though that we can not rule out the possibility that unknown indirect *in vivo* PKC stimulatory pathways might also be affected by the PIM87 mutation. The structural change underlying the lack of response of PIM87 to PKC is not readily apparent. After identifying PIM87, we generated several point mutants at or near amino acid 87, converting charged residues to non-charged residues, or changing small non-polar amino acids to proline, since the pentapeptide inserted contained a proline that would break the α-helix covering this site. However, none of the point mutations resulted in a PKC-non-responsive PLD, so it does not appear likely that the local region is literally part of the PKC-interaction site. Supporting this speculation, secondary structure analysis programs suggest that the region surrounding amino acid 87 is not on the surface of the protein.

The interaction of PKC with PLD1 versus the stimulation of PLD1 by PKC is not well understood. Stimulation involves the regulatory domain of PKC (Singer *et al*., 1996; S.M.Hammond and A.J.Morris, manuscript submitted). Physical contacts are most likely to involve the PKC catalytic and regulatory domains interacting with PLD1 both within and outside the N-terminal 325 amino acid region (Sung *et al*., 1999b). Accordingly, the PIM87 mutation is most likely to affect PLD1 stimulation by PKC through a subtle change to the protein structure at a site remote from amino acid 87, but this does not necessarily signify a loss of physical association between PLD1 and PKC. In examination of this issue, we have been unable to detect a difference in association of wild-type PLD1 or PIM87 with PKCα (our unpublished data).

Analysis of signaling using a PKC-non-responsive PLD1 allele

Our results (Figure 7) suggest that direct activation by PKC plays a vital role in the stimulation of PLD1 during agonist signaling. This is the first study to address this issue using a genetic tool instead of pharmacological inhibitors. Accordingly, this is the first time in which the role of PKC in PLD stimulation has been examined without the confounding factor of all of the other cellular pathways regulated by PKC being simultaneously affected. Moreover, even leaving this issue aside, as discussed

above it is now clear that the inhibitors used to inhibit PKC are not adequate for addressing the issue of PLD activation. First, since PKC activates PLD1 through its regulatory domain in a nucleotide-independent manner (Hammond *et al*., 1997), catalytic inhibitors of PKC work in some manner different from blocking direct PLD1 stimulation by PKC (Houle and Bourgoin, 1999). Secondly, the widely used regulatory PKC inhibitor calphostin is also useless in this setting, since it is as potent an inhibitor of PLD activity as it is of PKC activation (S.M.Hammond and A.J.Morris, manuscript submitted). Thirdly, most inhibitors are eventually recognized not to be as selective as initially reported. Calphostin is one example. Bisindolylmaleimide, which has been widely used as a PKC inhibitor but is now appreciated to inhibit muscarinic receptors directly, is another (Lazareno *et al*., 1998).

Rho signaling has been proposed to play a vital role in PLD signaling (Rumenapp *et al*., 1998). Much of this work involves bacterial protein toxin inhibitors and has recently been shown to be problematic, in part because Rho has many vital downstream effector pathways that indirectly affect PLD stimulation by any effector. For example, and one of the most serious, Rho inhibitors block synthesis of PIP_2 (Schmidt *et al*., 1996; Rumenapp *et al*., 1998), which is a co-factor required for PLD1 to be active (Hammond *et al*., 1995). We found that PIM87 does respond to RhoA under non-stimulated conditions (Figure 5B) and in the setting of agonist stimulation (Figure 7C), although we can not presently determine whether this response is direct or indirect (for example, through Rho kinase; Schmidt *et al*., 1999). Addressing that issue will require Rho-insensitive PLD1 alleles.

Regardless of whether the response is direct or indirect, however, it is likely that RhoA synergizes with PKC to stimulate PLD1 in *vivo*, raising the question of why these pathways converge ultimately to generate increased levels of PA in perinuclear membranes and vesicles through the action of PLD1. Rho has also been proposed to synergize with ARF in stimulating PLD during fMLP signaling (Fensome *et al*., 1998) and muscarinic signaling (Rumenapp *et al*., 1995), suggesting that full PLD1 activation may come about only when all three classes of effectors coincide, although there are other reports that have failed to demonstrate a role for ARF (Meacci *et al*., 1999).

Finally, we have only examined several examples of G-protein-coupled PLD1 signaling in a single type of cell. A more extensive survey of receptors and cells, and in particular, examination of the role of PKC in receptor tyrosine kinase signaling, will be required to generalize the findings reported here.

Materials and methods

General reagents

[^{3}H]dipalmitoyl phosphatidylcholine [choline-methyl-^{3}H] ([^{3}H]PC) was obtained from NEN Life Science Products (Boston, MA), and palmitic acid [9,10-3H(N)] was obtained from American Radiolabeled Chemicals, Inc. (St Louis, MO). GTPγS and anti-HA monoclonal antibody (clone 12CA5) were purchased from Roche (Indianapolis, IN). Thin layer chromatography (TLC) plates (LK5DF silica gel 150A or K6 silica gel 60A) were obtained from Fisher (Pittsburgh, PA). All cell culture media [i.e. Dulbecco's modified Eagle's medium (DMEM), Opti-MEM-I and Ham's F10 media] and LipofectAmine PLUS were from Gibco-BRL (Gaithersburg, MD). Anti-mouse IgG from goat conjugated with horseradish peroxidase was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). PIP_2, carbachol and PMA were from Sigma (St Louis, MO), all the other lipids were from Avanti Polar Lipids (Alabaster, AL). Bombesin was from Calbiochem (La Jolla, CA). All other reagents were of analytical grade unless otherwise specified.

Plasmids and bacteria strains

Wild-type and mutant PLD1 coding sequences were cloned into the mammalian expression vector pCGN, which contains a CMV promoter and encodes an N-terminal appended HA-epitope tag (Hammond *et al*., 1995). Before carrying out the transposon insertion mutagenesis protocol, the sole *Kpn*I site present in pCGN-hPLD1b was destroyed by altering a wobble codon using site-directed mutagenesis. This plasmid, denoted pCGN-hPLD1-K$^-$, was used as the wild-type control for all of the experiments reported herein. Plasmid pCD-PS hm3, which encodes the m3 muscarinic acetylcholine receptor, was kindly provided by Dr Martina Schmidt. The m1 muscarinic receptor and bombesin receptor plasmids were obtained from Onyx (Bradenton, FL). pEF-BOS-HA-RhoA and pEF-BOS-HA-RhoA^{Val14} were kindly provided by Dr Yasunori Kanaho. Pentapeptide mutagenesis bacterial strains and plasmids were kind gifts of Dr Finbarr Hayes.

Pentapeptide insertion mutagenesis

The pentapeptide scanning mutagenesis was performed as published (Hayes *et al*., 1997) with the following modification. A pool of PLD1 expression plasmids randomly interrupted once each by the transposon Tn4430Ω5 were generated by co-transformation of pCGN-hPLD1-K$^-$ and the transposon encoding plasmid pHT385 followed by a mass plasmid preparation from ~6000 bacterial colonies containing resolved plasmids. The plasmid pool was restricted using *Xba*I and *Xma*I, which released the PLD1 cDNA from pCGN. The DNA fragments were then electrophoresed to separate hPLD1 cDNAs containing transposons (7 kb) from wild-type hPLD1 (3 kb), wild-type pCGN (5 kb) and pCGN containing transposons (9 kb). The hPLD1-transposon fragments were isolated, re-cloned into fresh pCGN at the *Xba*I and *Xma*I sites, transformed into XL1-blue bacterial cells, and harvested to generate a new pool of plasmids. Finally, the transposons were excised by restricting the plasmid pool with *Kpn*I, which releases the transposon but leaves behind a residual of 15 bp, recircularized, transformed into XL1-blue and recovered as single colonies for subsequent characterization.

Cell culture and transfections

COS-7 and HEK293 cells were maintained in complete media [DMEM with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin] in a humidified atmosphere containing 5% CO_2 at 37°C. Transient transfections using LipofectAmine or LipofectAmine PLUS were carried out as described previously (Hammond *et al*., 1995; Colley *et al*., 1997; Sung *et al*., 1997, 1999b).

PLD activity assays

PLD activity assays were carried out using the *in vitro* head-group release assay for a 30 min time period and the *in vivo* transphosphatidylation assay (Morris *et al*., 1997) as previously described (Hammond *et al*., 1995; Colley *et al*., 1997; Sung *et al*., 1997, 1999b). Recombinant ARF1, RhoA and PKCα and -β were purified and activated using 50 μM GTPγS or 100 nM PMA as previously described (Hammond *et al*., 1997; Liang and Kornfeld, 1997). ATP was not present in the assay when PKC was used.

Western blotting and subcellular fractionation

Recombinant protein was visualized by ECL Western blotting analysis using the anti-HA-tag monoclonal antibodies 12CA5 or 3F10 as previously described (Sung *et al*., 1997). The relative amounts of protein expressed are difficult to quantitate because PLD1 has a tendency to become trapped at the interface between the stacking and resolving gels to a variable extent (Hammond *et al*., 1997). For the subcellular fractionation, COS-7 cell lysates were centrifuged at 3000 *g* for 5 min at 4°C to remove nuclei and unbroken cells and then the supernatant was further centrifuged at 30 000 *g* for 30 min at 4°C. The supernatant was collected as the cytoplasmic fraction, and the pellet resuspended into the equivalent volume of loading buffer by sonication as the membrane fraction.

Baculovirus expression of PLD proteins

The recombinant baculovirus harboring the PLD1 wild-type and mutant cDNAs was generated, selected and propagated using standard methods,

and PLD1 proteins or membranes containing the PLD1 proteins were prepared as described previously (Hammond *et al*., 1995, 1997).

Acknowledgements

The authors thank F.Hayes for assistance with the pentapeptide mutagenesis, T.-C.Sung for suggesting the scheme, Y.Kanaho for the gift of pEF-BOS RhoA, J.Engebrecht, S.Tsirka, V.Sciorra and members of the laboratory for critical reading of the manuscript and discussion, and C.Liang for exploring additional unpublished experiments. This work was supported by grants from Onyx Pharmaceutical Inc. and the NIH to M.A.F. (GM54813) and A.J.M. (GM50388).

References

BoyanoAdanez,M.C., Lundqvist,C., Larsson,C. and Gustavsson,L. (1997) Characterization of phospholipase D activation by muscarinic receptors in human neuroblastoma SH-SY5Y cells. *Neuropharm*., **36**, 295–304.

Briscoe,C.P., Martin,A., Cross,M. and Wakelam,M.J. (1995) The roles of multiple pathways in regulating bombesin-stimulated phospholipase D activity in Swiss 3T3 fibroblasts. *Biochem. J*., **306**, 115–122.

Brown,H.A., Gutowski,S., Moomaw,C.R., Slaughter,C. and Sternweis,P.C. (1993) ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell*, **75**, 1137–1144.

Colley,W., Sung,T.C., Roll,R., Hammond,S.M., Altshuller,Y.M., Bar-Sagi,D., Morris,A.J. and Frohman,M.A. (1997) Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr. Biol.*, **7**, 191–201.

Fensome,A., Whatmore,J., Morgan,C., Jones,D. and Cockcroft,S. (1998) ADP-ribosylation factor and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. *J. Biol. Chem.*, **273**, 13157–13164.

Frohman,M.A., Sung,T.-C. and Morris,A.J. (1999) Phospholipase D structure and regulation. *Biochim. Biophys. Acta*, **1439**, 175–186.

Hammond,S.M., Altshuller,Y.M., Sung,T.-C., Rudge,S.A., Rose,K., Engebrecht,J., Morris,A.J. and Frohman,M.A. (1995) Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J. Biol. Chem.*, **270**, 29640–29643.

Hammond,S.M., Jenco,J.M., Nakashima,S., Cadwallader,K., Cook,S., Nozawa,Y., Frohman,M.A. and Morris,A.J. (1997) Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by PIP2, ARF, and Rho family monomeric G-proteins and protein kinase C-α. *J. Biol. Chem.*, **272**, 3860–3868.

Hayes,F., Hallet,B. and Cao,Y. (1997) Insertion mutagenesis as a tool in the modification of protein function. Extended substrate specificity conferred by pentapeptide insertions in the Ω-loop of TEM-1 β-lactamase. *J. Biol. Chem.*, **272**, 28833–28836.

Hippenstiel,S., Kratz,T., Krull,M., Seybold,J., Eichel-Streiber,C. and Suttorp,N. (1998) Rho protein inhibition blocks protein kinase C translocation and activation. *Biochem. Biophys. Res. Commun*., **245**, 830–834.

Hou,W., Tsuda,T. and Jensen,R.T. (1998) Neuromedin B activates phospholipase D through both PKC-dependent and PKC-independent mechanisms. *Biochim. Biophys. Acta*, **1391**, 337–350.

Houle,M.G. and Bourgoin,S. (1999) Regulation of phospholipase D by phosphorylation-independent mechanisms. *Biochim. Biophys. Acta*, **1439**, 135–150.

Jones,D., Morgan,C. and Cockcroft,S. (1999) Phospholipase D and membrane traffic. *Biochim. Biophys. Acta*, **1439**, 229–244.

Keller,J., Schmidt,M., Hussein,B., Rumenapp,U. and Jakobs,K.H. (1997) Muscarinic receptor-stimulated cytosol-membrane translocation of RhoA. *FEBS Lett.*, **403**, 299–302.

Ktistakis,N.T. (1998) Signalling molecules and the regulation of intracellular transport. *BioEssays*, **20**, 495–504.

Lazareno,S., Popham,A. and Birdsall,N.J.M. (1998) Muscarinic interactions of bisindolylmaleimide analogues. *Eur. J. Pharm*., **360**, 281–284.

Lee,C. *et al*. (1997) Inhibition of phospholipase D by clathrin assembly protein 3 (AP3). *J. Biol. Chem.*, **272**, 15986–15992.

Liang,J.O. and Kornfeld,S. (1997) Comparative activity of ARF family members in the early steps of coated vesicle formation on rat live Golgi membranes. *J. Biol. Chem.*, **272**, 4141–4148.

Mamoon,A.M., Smith,J., Baker,J. and Farley,J.M. (1999) Activation of muscarinic receptors in porcine airway smooth muscle elicits a transient increase in phospholipase D activity. *J. Biomed. Sci.*, **6**, 97–105.

Manifava,M., Sugars,J. and Ktistakis,K.T. (1999) Modification of catalytically active phospholipase D1 with fatty acid *in vivo*. *J. Biol. Chem.*, **274**, 1072–1077.

Meacci,E., Vasta,V., Moorman,J.P., Bobak,D.A., Bruni,P., Moss,J. and Vaughan,M. (1999) Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A459 cells. *J. Biol. Chem.*, **274**, 18605–18612.

Min,D.S., Kim,E.C. and Exton,J.H. (1998) Involvement of tyrosine phosphorylation and protein kinase C in the activation of phospholipase D by H_2O_2 in Swiss 3T3 fibroblasts. *J. Biol. Chem.*, **273**, 29986–29994.

Morris,A.J., Frohman,M.A. and Engebrecht,J. (1997) Measurement of phospholipase D activity. *Anal. Biochem.*, **252**, 1–9.

Park,S.-K., Provost,J.J., Bae,C.D., Ho,W.-T. and Exton,J.H. (1997) Cloning and characterization of phospholipase D from rat brain. *J. Biol. Chem.*, **272**, 29263–29271.

Rose,K., Rudge,S.A., Frohman,M.A., Morris,A.J. and Engebrecht,J. (1995) Phospholipase D signaling is essential for meiosis. *Proc. Natl Acad. Sci. USA*, **92**, 12151–12155.

Rumenapp,U., Geiszt,M., Wahn,F., Schmidt,M. and Jakobs,K.H. (1995) Evidence for ADP-ribosylation-factor-mediated activation of phospholipase D by M3 muscarinic acetylcholine receptor. *Eur. J. Biochem*., **234**, 240–244.

Rumenapp,U., Schmidt,M., Olesch,S., Ott,S., von Eichel-Streiber,C. and Jakobs,K.H. (1998) Tyrosine-phosphorylation-dependent and Rho-protein-mediated control of cellular phosphatidylinositol 4,5-bisphosphate levels. *Biochem. J*., **334**, 625–631.

Schmidt,M., Rumenapp,U., Bienek,C., Keller,J. and von Eichel-Streiber,C. (1996) Inhibition of receptor signaling to phospholipase D by *Clostridium difficile* toxin B. Role of Rho proteins. *J. Biol. Chem.*, **271**, 2422–2426.

Schmidt,M. *et al*. (1998) Specific inhibition of phorbol ester-stimulated phospholipase D by *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B-1470 in HEK-293 cells—restoration by Ral GTPases. *J. Biol. Chem.*, **273**, 7413–7422.

Schmidt,M., Voss,M., Oude Weernink,P.A., Wetzel,J., Amano,M., Kaibuchi,K. and Jakobs,K.H. (1999) A role for Rho-kinase in Rho-controlled phospholipase D stimulation by the m3 muscarinic acetylcholine receptor. *J. Biol. Chem.*, **274**, 14648–14654.

Sciorra,V.A., Rudge,S.A., Prestwich,G.D., Frohman,M.A., Engebrecht,J. and Morris,A.J. (1999) Identification of a phosphoinositide binding motif that mediates activation of mammalian and yeast phospholipase D isoenzymes. *EMBO J.*, **18**, 5911–5921.

Singer,W.D., Brown,H.A., Jiang,X. and Sternweis,P.C. (1996) Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. *J. Biol. Chem.*, **271**, 4504–4510.

Sung,T.-C. *et al*. (1997) Mutational analysis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. *EMBO J*., **16**, 4519–4530.

Sung,T.-C., Altshuller,Y.M., Morris,A.J. and Frohman,M.A. (1999a) Molecular analysis of mammalian phospholipase D2. *J. Biol. Chem.*, **274**, 494–502.

Sung,T.-C., Zhang,Y., Morris,A.J. and Frohman,M.A. (1999b) Structural analysis of human phospholipase D1. *J. Biol. Chem.*, **274**, 3659–3666.

Toda,K., Nogami,M., Murakami,K., Kanaho,Y. and Nakayama,K. (1999) Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes. *FEBS Lett*., **442**, 221–225.

Watanabe,N. *et al*. (1997) p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J*., **16**, 3044–3056.

Yamazaki,M. *et al*. (1999) Interaction of the small G protein RhoA with the C-terminus of human phospholipase D1. *J. Biol. Chem.*, **274**, 6035–6038.

Yeo,E.J. and Exton,J.H. (1995) Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. *J. Biol. Chem.*, **270**, 3980–3988.

Received June 21, 1999; revised September 20, 1999; accepted September 29, 1999