Mechanisms of Regulation of Phospholipase D1 and D2
by the Heterotrimeric G proteins G_{13} and G_q

Zhi Xie#, Wan-Ting Ho#, Rachel Spellman, Songmin Cai and John H. Exton*

Howard Hughes Medical Institute and
Department of Molecular Physiology and Biophysics
Vanderbilt University School of Medicine
Nashville, TN 37232-0295

#These two authors contributed equally to this paper.

*Investigator of the Howard Hughes Medical Institute to whom all correspondence should be addressed. Telephone No. (615) 322-6494, FAX No. (615) 322-4381, E-mail john.exton@mcmail.vanderbilt.edu

Running Title: Activation of Phospholipase D by G_{13} and G_q

Key words: Phospholipase D, G Protein, Protein Kinase C, Rho, G_q, G_{13}, Phosphorylation, Palmitoylation
ABSTRACT

Our earlier studies of rat brain phospholipase D1 (rPLD1) showed that the enzyme could be activated in cells by α subunits of the heterotrimeric G proteins G₁₃ and G₉. Recently, we showed that rPLD1 is modified by Ser/Thr phosphorylation and palmitoylation. In this study, we first investigated the roles of these post-translational modifications on the activation of rPLD1 by constitutively active G₉₁₃Q226L and G₉₀₉₂₀₉L. Mutations of Cys²⁴⁰ and Cys²⁴¹ of rPLD1, which abolish both post-translational modifications, did not affect the ability of either G₉₁₃Q226L or G₉₀₉₂₀₉L to activate rPLD1. However, the RhoA-insensitive mutants, rPLD1(K946A, K962A) and rPLD1(K962Q), were not activated by G₉₁₃Q226L, although these mutant enzymes responded to phorbol ester and G₉₀₉₂₀₉L. On the contrary, the PKC-insensitive mutant rPLD1(ΔN168), which lacks the first 168 amino acids of rPLD1, responded to G₉₁₃Q226L but not to G₉₀₉₂₀₉L. In addition, we found that rPLD2 was strongly activated by G₉₀₉₂₀₉L and phorbol ester. However, surprisingly, the enzymatic activity of rPLD2 was suppressed by G₉₁₃Q226L and constitutively active V¹⁴⁴RhoA in COS 7 cells. Abolition of the post-translational modifications of rPLD2 did not alter the effects of G₉₀₉₂₀₉L or G₉₁₃Q226L. The suppressive effect of G₉₁₃Q226L on rPLD2 was reversed by dominant negative N¹⁹RhoA and the C3 exoenzyme of C. botulinum, further supporting a role for RhoA. In summary, G₉₁₃ activation of rPLD1 in COS 7 cells is mediated by Rho, while G₉₀₉ activation requires
PKC. rPLD2 is activated by $G_{Qq}$, but is inhibited by $G_{Qq13}$. Neither Ser/Thr phosphorylation nor palmitoylation is required for these effects.
INTRODUCTION

Phospholipase D (PLD)\(^1\) is a ubiquitous enzyme that hydrolyzes phosphatidylcholine to phosphatidic acid and choline (1). Phosphatidic acid is generally recognized as the signaling product of PLD action, but it can be converted to diacylglycerol or lysophosphatidic acid. Although the physiological role of PLD remains unclear, multiple functions have been proposed based on the actions of phosphatidic acid and its derivatives, and the involvement of PLD in signaling pathways (2,3).

PLD is highly regulated and responds to many growth factors, hormones and agonists (1,2). The responses are mediated by either tyrosine kinase- or heterotrimeric G protein-coupled receptors. For example, epidermal growth factor and platelet-derived growth factor function through receptor tyrosine kinases and require downstream small G proteins and PKC for the stimulation of PLD activity (4,5). Receptors linked to heterotrimeric G proteins of the G\(_q\) and G\(_i\) families have also been shown to induce PLD activation in many cell types (1,6), while G\(_{12/13}\) proteins have been shown to be involved in PLD stimulation by M3 muscarinic acetylcholine receptors in HEK293 cells (7,8).

To date, two isoforms of mammalian PLD (PLD1 and PLD2) have been cloned. These isoforms share about 50% amino acid similarity, but exhibit quite different regulatory properties. PLD1 has a low basal activity and responds to PKC and members of the Rho, ARF and Ras/Ral families of small G proteins (9-14). PLD2, on the other hand, exhibits a high basal activity and shows little or no response to stimuli compared to PLD1 (15-17). However, a recent study has
shown that PLD2 can be tyrosine phosphorylated and its activity up-regulated in HEK293 cells treated with epidermal growth factor (18). In addition, exogenously expressed PLD2 in HEK293 cells has been shown to be stimulated by insulin (19). The precise cellular localization of the two isoforms is not well defined, although PLD1 is generally believed to be located at the perinuclear region, while PLD2 is at plasma membrane (20).

Characterization of PLD1 and PLD2 has defined multiple functional domains and interesting properties of the enzymes. PLD belongs to a superfamily defined by the “HKD” motif HxK(x)₄D (21-23). PLD contains two copies of the HKD motif located in the N- and C-terminal halves of the molecule and both motifs are required for the enzymatic activity (24-25). Our studies on rat PLD1 (rPLD1) showed that there is an interdomain association between the N- and C-terminal halves of the molecule and this is important for the catalytic activity (25-26).

Another domain essential for the catalytic activity of PLD is located at the C-terminus of the enzyme, and deletion of the last amino acid of rPLD1 abolishes its enzymatic activity (27-28, 30). Besides the motifs essential for the catalysis, domains important for the PKC and Rho responses have been mapped to the N-terminal and C-terminal regions of the enzyme, respectively (29-32). In addition, recent studies of rPLD1 in COS 7 cells have revealed that the molecule is modified by Ser/Thr phosphorylation (26) and palmitoylation (33-34). These two post-translational modifications are interrelated and require association between the N- and C-terminal halves of rPLD1 and the presence of the N-terminal amino acids of the enzyme (33).

Although neither palmitoylation nor phosphorylation was found to be essential for the catalysis of rPLD1(33), the modifications played roles in specifying the cellular location of the enzyme (34) and strengthening its association with membranes (33). Earlier studies of rPLD1
expressed in COS 7 cells showed that it was stimulated by constitutively active \( G_{\alpha 13} \) and \( G_{\alpha q} \) (35). Here we investigated the effects of these activated \( \alpha \)-subunits on rPLD1 and rPLD2 and tested whether the Ser/Thr phosphorylation or palmitoylation of the enzymes play roles in the effects. In addition, we used rPLD1 mutants that are unable to respond to PKC or to Rho to dissect the downstream effectors that mediate the effects of \( G_{\alpha 13} \) and \( G_{\alpha q} \).
EXPERIMENTAL PROCEDURES

Materials – 4β-Phorbol 12-myristate 13-acetate (PMA), bovine serum albumin, Triton X-100 and microcystin were from Sigma. Phosphatidylbutanol (PtdBut) standard was from Avanti Polar Lipids Corp. [3H]Myristic acid was from NEN Life Science Products. Protein A-agarose beads, Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were from Life Technologies, Inc. The transfection reagent FuGENE6 and the protease inhibitor cocktail were from Roche Molecular Biochemicals. COS7 cells were from American Type Culture Collection. SDS-polyacrylamide gels were from Novex. The PcDNA3 vectors and the monoclonal antibodies against the V5 and Xpress epitope tags were from Invitrogen. The constructs for V14RhoA and N19RhoA were gifts from A. Couvillon (Vanderbilt University). RhoA, Gαq and Gα13 antibodies were from Santa Cruz, and anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were from Vector Laboratories. C3 exoenzyme from C. botulinum was from Cytoskeleton, Denver CO. The Rho kinase inhibitor Y-27632 was kindly provided by Yoshitomi Pharmaceutical Industries Ltd., Saitama, Japan.

Plasmid Construction – N-terminal Xpress-tagged full length or truncated rPLD1 was created by PCR amplification and subcloned (11). The rPLD2 clone was obtained by PCR amplification using a rat brain quick cDNA mix (Clontech) and the PCR product was subcloned in frame with the N-terminal Xpress tag in PcDNA3 vector. The deletion or site-directed mutations of rPLD1 were generated as described in the QuickChange™ Site-Directed
Mutagenesis Instruction manual from Stratagene. All the constructs were sequenced to verify the coding regions of rPLD1. The PKC-insensitive and RhoA-insensitive rPLD1 mutants, rPLD1 (K946A, K962A) and rPLD1 (K962Q) were generated as described (25,32). Constitutive active Gα13 Q226L and GαqQ209L were generated as described (35).  

**Cell Culture and Transfection** – COS-7 cells were maintained in DMEM supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS in humidified 10% CO₂. Six-well plates were seeded with 2 x 10⁵ COS 7 cells per well and 10 cm-dishes were seeded with 6 x 10⁵ cells and transfected with FuGENE6 according to the manufacturer’s instructions.  

**In vivo PLD Assay** – After 6 h transfection with FuGENE6, COS 7 cells in 6-well plates were serum-starved (0.5% FBS in DMEM) in the presence of 1 µCi/ml [³H]myristic acid. After overnight starvation, the cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free medium supplemented with 0.3% bovine serum albumin for 50 min. PLD activity was then assayed as described (25). Briefly, cells were incubated in 0.3% 1-butanol for 25 min. Cells were then washed with ice-cold PBS and stopped with methanol. Lipids were extracted, and the PtdBut product was resolved by thin layer chromatography. Bands co-migrating with a PtdBut standard were quantitated by scintillation counting. The results are as the percentage of total lipid radioactivity that is incorporated into PtdBut.  

**Western Blotting** – Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The blots were then blocked with 5% non-fat milk and
incubated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescence.

Scrape Loading with C3 Exoenzyme – After 6 h transfection, cells growing on 100 mm dishes were washed with 5 ml of PBS and then with 2 ml of scraping buffer (114 mM KCl, 15 mM NaCl, 5.5 mM MgCl₂, 10 mM Tris-HCl). Cells were gently scraped in 0.5 ml of scraping buffer containing 0 or 5 mg/ml C3 exoenzyme. After brief centrifugation, cells were resuspended in DMEM containing 10% fetal bovine serum and split into 6-well plates. Following 24 h of culture, the scrape-loaded cells were serum-starved and labeled with [³H]myristic acid overnight for PLD assay.
RESULTS

rPLD1 Activation by Gα13 Requires Rho, while Activation by Gαq Requires PKC -

Earlier studies utilizing expression in COS7 cells have shown that rPLD1 can be stimulated by the constitutively active α-subunits of the heterotrimeric G proteins G13 \((G\alpha_{13}^{Q226L})\) and Gq \((G\alpha_{q}^{Q209L})\) (35). The activation of rPLD1 by Gα13Q226L was suppressed when the cells were treated with C3 exoenzyme from \textit{Clostridium botulinum} or when they were cotransfected with dominant negative RhoA (35), indicating that activation of rPLD1 by Gα13 required RhoA.

The effector that mediates the activation of rPLD1 by Gαq was not explored in (35). A likely candidate is PKC, since it is downstream of phosphoinositide phospholipase C (PLC) which is activated by Gαq stimulation. Molecular analysis of rPLD1 has localized the motifs for PKC and Rho responses at the N- and C-terminal halves of the enzyme, respectively (29-32).

In the present study, we used several rPLD1 mutants that have specifically lost responses to PKC or Rho to dissect the downstream effectors that mediate the activation of rPLD1 by Gα13 and Gαq. rPLD1(ΔN168), which lacks the first 168 amino acids, does not respond to PKC but is able to respond to Rho activation (29). On the other hand, certain site-directed rPLD1 mutants, namely rPLD1(K946A, K962A) and rPLD1(K962Q), are insensitive to Rho stimulation, but can be activated by PKC (32).

We first examined the effects of constitutively active Gα13Q226L on wild type rPLD1 and mutant rPLD1s that are unresponsive to Rho activation. These rPLD1 constructs were either
transfected alone or cotransfected with $G_{\alpha}13Q226L$ into COS 7 cells and in vivo PLD activity was measured. As shown in Fig. 1A, wild type rPLD1 was effectively activated by $G_{\alpha}13Q226L$. However, rPLD1(K946A, K962A) and rPLD1(K962Q), which are unresponsive to Rho (32), were not stimulated by $G_{\alpha}13Q226L$. These mutants were well expressed in comparison to wild-type rPLD1 (Fig. 1B). Although they showed lower basal activity (Fig. 1A and 1C), they responded fully to PMA stimulation (Fig. 1C and Ref. 32). Furthermore, when these Rho-insensitive mutants were examined for their response to co-transfected constitutively active $G_{\alpha}qQ209L$, they all responded strongly (data not shown). In agreement with previous findings (35), $G_{\alpha}13Q226L$ had no effect on the endogenous PLD of COS 7 cells (data not shown).

We next examined the effects of $G_{\alpha}13Q226L$ and $G_{\alpha}qQ209L$ on rPLD1(ΔN168). This N-terminal deletion mutant of rPLD1 does not respond to PMA (PKC) stimulation, but retains the ability to respond to RhoA (29). As shown in Fig. 2A, although this mutant had higher basal activity than wild type enzyme (25,29), it was further stimulated when cotransfected with $G_{\alpha}13Q226L$ in COS 7 cells. The higher activity of rPLD1(ΔN168) and its enhanced response to $G_{\alpha}13Q226L$ could not be attributed to increased expression (Fig. 2B). Constitutively active $G_{\alpha}qQ209L$ had a small effect on endogenous PLD and markedly stimulated wild type rPLD1 (Fig. 3A), but unmutated $G_{\alpha}q$ was ineffective (data not shown). However, minimal stimulation was observed when rPLD1(ΔN168) was cotransfected with $G_{\alpha}qQ209L$ (Fig. 3A). The rPLD1(ΔN168) constructs were expressed to a similar extent (Fig. 3B). The stimulation of wild type rPLD1 by $G_{\alpha}qQ209L$ was non-additive with that induced by PMA (data not shown), consistent with
their mediation by PKC.

In summary, the foregoing results showed that rPLD1 activation by Gα13 was dependent on the interaction of the enzyme with the small G protein Rho, while activation by Gαq did not require this. However, the N-terminal 168 amino acids which contain a PKC interaction site(s) were required for the activation of rPLD1 by Gαq, but not Gα13.

**Neither Ser/Thr Phosphorylation nor Palmitoylation of rPLD1 is Required for Gα13 or Gαq Activation** - Recent studies of PLD1 have shown that the enzyme can be modified by Ser/Thr phosphorylation and palmitoylation. Although neither modification was essential for catalysis (26,33), they played roles in specifying the cellular location of the enzyme (34) and strengthening its association with membranes (26,33). We thus examined whether these post-translational modifications played specific roles in the activation of rPLD1 by Gα13 or Gαq.

We utilized the double cysteine mutant, rPLD1(C241A, C242A), which lacks palmitoylation and phosphorylation. Our previous study showed that this mutant retained the ability to respond to PKC activation in vivo (33). The mutant rPLD1 was transfected alone or cotransfected with Gα13Q226L or GαqQ209L and the PLD activity measured. As shown in Fig. 4A, rPLD1(C241A, C242A) had dramatically reduced activity as compared with wild type enzyme, but the enzyme was well expressed (Fig. 4B) and responded to PMA stimulation (33 and data not shown).

When cotransfected with Gα13Q226L or GαqQ209L, the double cysteine mutant responded markedly to stimulation by both α-subunits (Fig. 4A and 5A). Indeed, the fold response of rPLD1(C241A, C242A) to Gα13Q226L or GαqQ209L was greater than wild type enzyme. Both
rPLD1 and rPLD1 (C241A, C242A) were expressed at comparable levels when transfected alone or cotransfected with the G protein α-subunits (Fig. 4B and 5B). Thus, neither Ser/Thr phosphorylation nor palmitoylation of rPLD1 is required for the activation of the enzyme by Gα13 or Gαq.

rPLD2 is Activated by Gαq and Phorbol Ester, but is Suppressed by Gα13 – PLD2 has quite different properties from PLD1 (15-19). PLD2 has a high basal activity and shows no responses to PKC, ARF and RhoA in vitro and modest responses to PKC and Arf in vivo (16,17). We next investigated the response of rPLD2 to constitutively active Gα13 and Gαq in COS 7 cells. As shown in Fig. 6A, 6B, 7A and 7B, both rPLD1 and rPLD2 were well expressed and showed large responses to GαqQ209L. When the amount of plasmid DNA transfected was varied, rPLD2 was consistently expressed to a greater extent than rPLD1 and showed a higher basal activity (Fig. 7A, 7B and data not shown). As previously noted (26, 33), rPLD1 showed an upper phosphorylated band on SDS-PAGE whereas rPLD2 migrated as a single band (Fig. 6B and 7B). Unexpectedly, co-expression of constitutively active Gαq enhanced the appearance of the lower band of rPLD1 (Fig. 6B and 7B), but we did not explore the basis for this. Both isozymes showed large responses to PMA, but there was little difference between the isozymes in the total activity observed in the presence of the phorbol ester (Fig. 7A). The increases with PMA were much greater than those observed with Gαq209L. Possible reasons for this are described in the Discussion.

A surprising observation was that, whereas rPLD1 was stimulated by Gα13Q226L (Figs. 1A, 2A and 4A), the activity of rPLD2 was suppressed when it was cotransfected with
The suppression of rPLD2 by Gα13Q226L was not due to lack of expression of the enzyme (Fig. 8B). A palmitoylation-deficient mutant of rPLD2 showed much loss of basal activity, but responded well to Gαq209L (Fig. 8A). The activity of the mutant rPLD2 was also suppressed by Gα13Q226L, although the effect was not large due to its already low activity.

**Role of RhoA in the Suppression of rPLD2 by Gα13** – In view of the evidence that Gα12/13 effects are mediated by RhoA (Fig. 1 and Refs 35-42), we tested to see if this small G protein was involved in the suppression of rPLD2 activity by Gα13Q226L. Figure 9A shows the effects of dominant negative N19RhoA on the action of Gα13Q226L. This RhoA mutant produced a small increase in rPLD2 activity, but more importantly, reversed the inhibition of PLD activity caused by Gα13Q226L. Dominant negative Rac1 was also tested, but had minimal effects (data not shown). To obtain further evidence for a role of RhoA, constitutively active V14RhoA was also tested, and this form of RhoA suppressed rPLD2 activity significantly (Fig. 10A). Figures 9B and 10B show that rPLD2, Gα13Q226L and RhoA were all well-expressed.

The C3 exoenzyme of Clostridium botulinum inactivates Rho proteins through ADP-ribosylation (36). This toxin reversed the inhibitory effect of Gα13Q226L on rPLD2 (Fig. 11A) and also abolished the effect of V14RhoA (data not shown). It had no effects on the expression of either rPLD2 or Gα13Q226L (Fig. 11B). To explore the possible role of Rho kinase in the actions of Gα13Q226L or RhoA on rPLD1 and rPLD2, we tested the effects of the Rho kinase
inhibitor Y-27632. This inhibitor significantly reduced the stimulatory effect of constitutively active $G_{\alpha}13$ on rPLD1 (data not shown). However, the inhibitor reduced the effect of PMA to a similar extent, raising questions about its specificity in this system. There was no consistent effect of Y-27632 to reverse the action of $G_{\alpha}13Q226L$ on rPLD2.
DISCUSSION

By using rPLD1 mutants that have specifically lost the ability to respond to PKC or Rho, we have dissected the factors involved in downstream signaling from G\textsubscript{\alpha13} and G\textsubscript{\alphaq} to this enzyme. We have provided further evidence that activation of rPLD1 by G\textsubscript{\alpha13} requires Rho (35) and have shown that activation by G\textsubscript{\alphaq} requires PKC. There are many reports that Rho can be stimulated by active G\textsubscript{\alpha13} (35-42), but there are data indicating that it can also be activated by G\textsubscript{\alphaq} (36,40,43,44). However, although G\textsubscript{\alphaq} has the potential to activate Rho, this did not lead to activation of rPLD1 in COS 7 cells (Fig. 3). As shown in this figure, rPLD1(ΔN168), which does not respond to PKC but retains the ability to respond to Rho, was not activated by G\textsubscript{\alphaq}.

The activation of rPLD2 by G\textsubscript{\alphaq}Q209L and PMA was unexpected in view of the report that this isozyme does not respond to PKC in vitro (16). However, there has been another recent report showing that PLD2 is activated by co-expression with PKC\textsubscript{α} and PKC\textsubscript{δ} in Sf9 cells (45). Furthermore, studies of human PLD2 have shown that the enzyme is activated by insulin treatment in HEK293 cells, and this activation is mediated by PLC and PKC (19). G\textsubscript{\alphaq} is well known to activate PLC which, in turn, leads to activation of PKC, and the data of Fig. 3 with N-terminally truncated rPLD1 support the idea that the activation of rPLD1 by G\textsubscript{\alphaq} is mediated by PKC. As expected from previous studies (11,12,25,26), PMA increased rPLD1 activity in COS 7 cells (Fig. 7A), but the increase was much larger than that observed with G\textsubscript{\alphaq}Q209L. This
presumably reflects the greater ability of the phorbol ester to activate PKC compared with
diacylglycerol generated endogenously through the activation of PLC. Another possibility is that
transfection of the cells for 24 h with constitutively active G$_{q}$q may have led to down-regulation
of PKC.

PMA activated rPLD2 to approximately the same extent as rPLD1 (Fig. 7A). This was
surprising in view of in vitro observations (12) and was not attributable to differences in the
expression of the PLD isozymes (Fig. 7B). Although several groups have demonstrated that
PLD1 can be activated by conventional isozymes of PKC through a non-phosphorylating
mechanism in vitro (10,46-50), it is possible that a different mechanism involving
phosphorylation is utilized by PKC in vivo. Whether or not the PLD isozymes themselves or
other interacting proteins are phosphorylated remains to be defined. Direct phosphorylation of
PLD1 by PKC$_{\alpha}$ in vitro does not result in activation (10,49). However, there have been reports
indicating that PLD1 is phosphorylated and activated by PKC in vivo (51,52). Our studies
indicate that neither palmitoylation nor the associated phosphorylation of either rPLD1 or rPLD2
significantly modify its response to G$_{q}$q in COS 7 cells (Fig. 5 and 8). Previous work has shown
that these post-translational modifications do not impair the response of either isozyme to PMA
(33,53).

It was surprising and interesting to find that rPLD2 activity was suppressed when
cotransfected with G$_{q}$13Q226L (Fig. 7-10). This was in marked contrast to rPLD1, which was
activated. rPLD2 differs from rPLD1 is that it is not extensively modified by Ser/Thr
phosphorylation, although it is palmitoylated (53). However, in this study we found that both
these post-translational modifications of rPLD1 or rPLD2 were not required for the effects of either $G_\alpha q$ or $G_\alpha 13$. Thus, lack of Ser/Thr phosphorylation is unlikely to be responsible for the different response of rPLD2 to the activation of $G_\alpha 13$.

Human PLD2 shows no change in catalytic activity when assayed in vitro in the presence of RhoA-GTPγS (16). Due to its high basal activity, any inhibitory effect of Rho should have been discernible. However, it is suppressed by constitutively active V$^{14}$RhoA when assayed in COS 7 cells, and the inhibitory effect of active $G_\alpha 13$ is reversed by dominant negative N$^{19}$RhoA. These findings indicate that Rho mediates the inhibition by $G_\alpha 13$. We were unable to implicate Rho kinase in the effects of V$^{14}$RhoA or $G_\alpha 13$ on either rPLD1 or rPLD2. Although an inhibitor of Rho kinase (Y-27632) reduced their stimulatory effects on rPLD1, a similar magnitude of inhibition was observed when the inhibitor was tested against PMA, indicating a lack of specificity in this system. With respect to the effects of the inhibitor on rPLD2, no consistent changes were observed.

As discussed above for PLD1, the in vivo effects of PKC on PLD2 could involve another protein(s) and this is also true for the action of RhoA on this isozyme. PLD2 has a much higher basal activity than PLD1 in vitro, but this difference is less evident in vivo (Fig. 6A and 7A and Ref. 16 and 45). This may be because there is suppression of PLD2 activity in intact cells (16). Several inhibitors of PLD2 have been reported. These include ($\alpha$- and $\beta$-synucleins (54), $\alpha$-actinin (55), $\beta$-actin (56) amphiphysins (57) and also proteins that reduce the cellular level of phosphatidylinositol 4,5-P$_2$ (60,61). However, most of these proteins also act on PLD1 and are
therefore unlikely to specifically suppress PLD2 or mediate a selective inhibitory effect of RhoA on this isozyme. RhoA has many cellular targets (36,60) but their possible links to PLD2 are presently unknown. Thus the intriguing observation that G\(\alpha\)13 and RhoA have opposite effects on PLD1 and PLD2 in vivo requires much further work to elucidate the mechanisms and functional significance.
REFERENCES


FIGURE LEGENDS

Fig. 1  Response of Rho-insensitive rPLD1 Mutants to Constitutively Active Gα13Q226L and Phorbol Ester.
Wild type or mutated rPLD1 was either transfected alone or cotransfected with constitutively active Gα13Q226L into COS 7 cells as indicated in the figure.  A, PLD activity was measured as described under "Experimental Procedures" and is represented by the incorporation of radioactivity into PtdBut as a percentage of that incorporated into the lipid of the cells.  Means ± S.E.M. values from three experiments performed in triplicate are shown.  B, a representative cell lysate was analyzed by PAGE on a 8% polyacrylamide gel followed by Western blotting with either anti-Xpress or anti-Gα13 antibodies.  C, rPLD1 or its Rho-insensitive mutants were transfected into COS 7 cells and the PLD activity was measured after treatment with 100 nM PMA for 20 min.  rPLD1, N-terminally Xpress-tagged rPLD1; Gα13QL, constitutively active Gα13Q226L.  rPLD1 (K946A, K962A) and rPLD1 (K962Q), Rho-insensitive mutants of rPLD1.

Fig. 2  Activation of PKC-insensitive Mutant, rPLD1(ΔN168), by Constitutively Active Gα13.
Wild type or N-terminally truncated mutant rPLD1(ΔN168), was either transfected alone or cotransfected with constitutively active Gα13Q226L into COS 7 cells as indicated in the figure.  A, PLD activity was measured as described in Fig. 1 legend.  Means ± S.E.M. values from three experiments performed in triplicate are shown.  B, a representative cell lysate was analyzed by Western blotting.  rPLD1(ΔN168), N-terminal Xpress-tagged rPLD1 lacking the N-terminal 168 amino acids.
Fig. 3  The PKC-insensitive Mutant, rPLD1 (N168) does not Respond to Constitutively Active
G_αq. Wild type or N-terminally truncated mutant, rPLD1(N168), was either transfected alone
or with constitutively active G_αqQ209L into COS 7 cells as indicated in the figure.  A, PLD
activity was measured as described in Fig. 1 legend.  Means ± S.E.M. from three experiments
performed in triplicate are shown.  B, a representative cell lysate was analyzed by Western
blotting with either anti-Xpress or anti-G_αq antibodies.  G_αqQL, constitutively active
G_αqQ209L.

Fig. 4 Activation of Palmitoylation-deficient rPLD1 Mutant by Constitutively Active G_α13.
Wild type or palmitoylation-deficient mutant, rPLD1(C241A, C242A), was either transfected
alone or cotransfected with constitutively active G_α13Q226L into COS 7 cells as indicated in the
figure.  A, PLD activity was measured as described in Fig. 1 legend.  Means ± S.E.M. values
from three experiments performed in triplicate are shown.  B, a representative cell lysate was
analyzed by Western blotting with either anti-Xpress or anti-G_α13 antibodies.  rPLD1(C240A,
C241A), palmitoylation-deficient rPLD1 mutant.

Fig. 5 Activation of Palmitoylation-deficient rPLD1 Mutant by constitutively Active G_αq.
Wild type or palmitoylation-deficient mutant rPLD1(C241A, C242A) was either transfected alone
or cotransfected with constitutively active G_αqQ209L into COS 7 cells as indicated in the figure.
A, PLD activity was measured as described in Fig. 1 legend.  A representative experiment of
two performed in triplicate is shown. B, a representative cell lysate was analyzed by Western blotting with either anti-Xpress or anti-Gαq antibodies.

**Fig. 6 Activation of rPLD1 and rPLD2 by Constitutively Active Gαq.** rPLD1 and rPLD2 were either transfected alone or cotransfected with constitutively active GαqQ209L into COS 7 cells as indicated in the figure. A, PLD activity was measured as described in Fig. 1 legend. Means ± S.E.M. from four experiments performed in triplicate are shown. B, a representative cell lysate was analyzed by Western blotting with either anti-Xpress or anti-Gαq antibodies.

**Fig. 7 Activation of rPLD1 and rPLD2 by Phorbol ester and Constitutively Active Gαq.** rPLD1 and rPLD2 were either transfected alone or cotransfected with constitutively active GαqQ209L into COS 7 cells and incubated with or without PMA as indicated in the figure. The amounts (µg) of plasmid DNA added were: rPLD1 a, 1.0; b, 1.6; rPLD2 a, 0.7; b, 1.1. Treatment with PMA (100 nM) was for 20 min. A, PLD activity was measured as described in Fig. 1 legend. A representative experiment of four performed in triplicate is shown. B, a representative cell lysate was analyzed by Western blotting with anti-Xpress or anti-Gαq antibodies.

**Fig. 8 Suppression of Wild Type or Palmitoylation-deficient rPLD2 Activity by Constitutively Active Gα13.** Wild type or palmitoylation-deficient rPLD2 (C223A, C224A) was either transfected alone or cotransfected with constitutively active Gα13Q226L or GαqQ209L into
COS 7 cells as indicated in the figure. A, PLD activity was measured as described in Fig. 1 legend. A representative experiment of two performed in triplicate is shown. B, a representative cell lysate was analyzed by Western blotting with either anti-Xpress, anti-\( \alpha_{13} \) and anti-\( \alpha_q \) antibodies. \( \text{rPLD2 (C223A, C224A)} \), palmitoylation-deficient rPLD2 mutant.

**Fig. 9** Reversal of Inhibitory Effect of Constitutively Active \( \alpha_{13} \) on rPLD2 by Dominant Negative \( \text{RhoA} \). rPLD2 was transfected alone or with dominant negative \( \text{N}^{19}\text{RhoA} \) or \( \alpha_{13}\text{Q226L} \) singly or together into COS 7 cells. A, PLD activity was measured as described in Fig. 1 legend. Means ± S.E.M. from five experiments performed in triplicate are shown. B, A cell lysate from a representative experiment was analyzed by Western blotting with anti-Xpress, anti-\( \alpha_{13} \) and anti-RhoA antibodies. \( \text{N}^{19}\text{RhoA} \), dominant negative RhoA.

**Fig. 10** Constitutively Active \( \text{RhoA} \) Suppresses rPLD2 Activity. rPLD2 was transfected alone or with constitutively active \( \alpha_{13}\text{Q226L} \) or constitutively active \( \text{V}^{14}\text{RhoA} \) into COS 7 cells. A, PLD activity was measured as described in Fig. 1 legend. Means ± S.E.M. from four experiments performed in triplicate are shown. B, a cell lysate from a representative experiments was analyzed by Western blotting with anti-Xpress, anti-\( \alpha_{13} \) and anti-RhoA antibodies. \( \text{V}^{14}\text{RhoA} \), constitutively active RhoA.

**Fig. 11** C3 Exoenzyme from \( \text{C. botulinum} \) Abolishes the Inhibitory Effect of Constitutively
**Active \( G_\alpha 13 \) on rPLD2 Activity.** rPLD2 was transfected with or without \( G_\alpha 13Q226L \) into COS 7 cells. Six h after transfection, the cells were washed and scraped off the plates in the absence or presence of 5 \( \mu \text{g/ml} \) C3 exoenzyme as described in (35).  

A, PLD was assayed as described in Fig. 1 legend. Means \( \pm \) S.E.M. from three experiments performed in triplicate are shown.  

B, a cell lysate from a representative experiment was analyzed by Western blotting with anti-Xpress and anti-\( G_\alpha 13 \) antibodies.

**ACKNOWLEDGEMENTS**

We wish to thank Judy Nixon for typing the manuscript.
1. The abbreviations used are: PLD, phospholipase D; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PMA, 4β-phorbol 12-myristate 13-acetate; PLC, phosphoinositide phospholipase C.
Fig. 1
Fig 1

PtdBut (Percentage of Total Incorporation)

- Vector
- rPLD1
- rPLD1(K962Q)
- rPLD1(K946A,K962A)

- PMA
+ PMA
Fig. 2
Fig. 3
**A**

PtdBut (Percentage of Total Incorporation)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PtdBut (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>0.1</td>
</tr>
<tr>
<td>rPLD1</td>
<td>0.2</td>
</tr>
<tr>
<td>rPLD1 + Gα13QL</td>
<td>0.5</td>
</tr>
<tr>
<td>rPLD1(C240A, C241A)</td>
<td>0.6</td>
</tr>
<tr>
<td>rPLD1(C240A, C241A) + Gα13QL</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**B**

Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 10
Fig. 11

A

PtdIns(Percentage of Total Incorporation)

- C3
+ C3

Vector rPLD2 rPLD2+Gα13QL

B

Western Blot

rPLD2 -
- C3 + C3 - C3 + C3

Anti-Xpress

Gα13QL —

Anti-Gα13

Downloaded from http://www.jbc.org/ by guest on November 19, 2017
Mechanisms of regulation of phospholipase D1 and D2 by the heterotrimeric G proteins G(13) and G(q)

Zhi Xie, Wan-Ting Ho, Rachel Spellman, Songmin Cai and John H. Exton

J. Biol. Chem. published online January 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M109751200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts