Stimulation of phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine (PtdCho) by phorbol 12-myristate 13-acetate (PMA) has been shown to be mediated by the α- and β-isozymes in the regulation of PLD-mediated phosphatidylethanolamine (PtdEtn) hydrolysis, MCF-7 human breast carcinoma cells overexpressing the α- and θ-isozymes, and R6 rat fibroblasts overexpressing the α-, β-, and ε-isozymes were used. In the vector control MCF-7 cells, which contain low levels of PKC-α, PMA (100 nM) had only small effects on the hydrolysis of PtdEtn (1.1–1.35-fold) and PtdCho (1.15–1.6-fold). Stable expression of PKC-α in MCF-7 cells, which was accompanied by increased levels of the β- and θ-isozymes as well, greatly enhanced both PMA-induced PLD-mediated formation of phosphatidylethanolamine (5-10-fold) and PtdEtn hydrolysis of PtdEtn (2.5–2.9-fold) and PtdCho (5.5–7.2-fold). The effects of PMA on the hydrolysis of PtdEtn (and PtdCho) in MCF-7/PKC-α cells were significantly inhibited by 0.5–3 µM concentrations of Gö 6976, a selective inhibitor of the conventional PKC subfamily. Stable expression of PKC-α in R6 fibroblasts enhanced, at a shorter (10 min) incubation time, the effects of PMA on the hydrolysis of both PtdEtn and, to a lesser extent, PtdCho. In contrast, stable expression of PKC-β in R6 fibroblasts, which originally did not contain this enzyme, enhanced the effects of PMA only on PtdCho, but not PtdEtn, hydrolysis. Overexpression of either PKC-θ in MCF-7 cells or PKC-ε in R6 and NIH 3T3 fibroblasts had no detectable effects on PMA-induced hydrolysis of PtdEtn. Collectively, the results suggest that PKC-α has a major role in the mediation of phorbol ester action on PtdEtn hydrolysis, while PtdCho hydrolysis may be regulated by both the α and β isozymes.

In many cell lines activators of phospholipase D (PLD),1

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1 The abbreviations used are: PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PtdOH, phospholipidic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MCF-7/PKC-α, MCF-7/PKC-β, MCF-7/PKC-δ and MCF-7/ε vector cells stably transfected with PKC-α, PKC-β, and the corresponding empty vectors, respectively; R6/PKC-α, R6/PKC-β, R6/PKC-ε and corresponding R6/vector cells stably transfected with the including the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA), appear to stimulate only the hydrolysis of phosphatidylethanolamine (PtdEtn) (1, 2). In Madin-Darby canine kidney cells (3) and in Swiss/3T3 cells (4) PKC-α was shown to be a major regulator of PtdEtn hydrolysis. However, expression of PKC-β (5) or addition of PKC-β to isolated membranes (6, 7) also enhanced the effect of PMA on PtdEtn hydrolysis. Interestingly, while PKC-α was a more effective mediator of PMA effect in lung fibroblast membranes than PKC-β (6), a reversed order of potency was observed in neutrophil membranes (7). Collectively, these data suggest that if expressed, both PKC-α and PKC-β may be able to regulate PtdCho hydrolysis.

The role of increased PtdCho hydrolysis in the mediation of cellular actions of PKC activators is unknown. Recently, we presented evidence showing that in NIH 3T3 fibroblasts PtdCho hydrolysis is unlikely to play a major role in the mediation of mitogenic effects of PMA (8). In fact, choline phosphate, which in agonist-treated cells can be formed by the sequential actions of PLD and choline kinase, and PMA were found to stimulate DNA synthesis by competitive mechanisms (9).

Ethanolamine (10), and particularly its methylated analogues (11, 12), can greatly enhance insulin-induced DNA synthesis by a wortmannin-sensitive mechanism not involving PtdEtn synthesis. PMA also potentiates the mitogenic effect of insulin by a wortmannin-sensitive mechanism (9). Moreover, in NIH 3T3 fibroblasts (13, 14) and in several other cell types (15–18) PMA can stimulate the formation of ethanolamine from phosphatidylethanolamine (PtdEtn) by a mechanism involving a specific PLD activity. These observations raise the possibility that ethanolamine may contribute to the growth regulating and/or tumor promoting effects of PMA.

Regulation of PtdEtn-hydrolyzing PLD by the PKC system is much less understood than regulation of PtdCho-specific PLD. Here, we set out to determine the role of various PKC isozymes in the mediation of PMA effect on PtdEtn hydrolysis. For this purpose, we used MCF-7 human breast carcinoma cells overexpressing PKC-α (19) or PKC-θ (20) and R6 rat fibroblasts overexpressing PKC-α (21), PKC-β (22), or PKC-ε (23), along with inhibitors of the various PKC isozymes. The results indicate that PKC-α has a major role in the mediation of stimulatory PMA effect on PtdEtn hydrolysis.

EXPERIMENTAL PROCEDURES

Materials—PMA, ethanolamine, choline, and Dowex 50W–H+ form were purchased from Sigma. GF 109203X and Gö 6976 were bought from Calbiochem. [1-14C]Palmitic acid (60 µCi/µmol), [2-14C]ethanolamine (50 µCi/µmol), [methyl-14C]choline chloride (50 µCi/µmol), corresponding PKC isozymes and empty vectors, respectively.

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and [methyl-\(^{3}H\)]thymidine (85 mCi/mmol) were bought from Amer- 7 bham, USA. Phosphatidylethanolamine (PtdEtOH) was from Avanti Polar Lipids Inc. Tissue culture reagents as well as affinity purified polynucleo- 47 tidespecific for PKC-\(\beta\) or PKC-\(\beta\)II were purchased from Life 336 Technologies, Inc. Biotinylated goat anti-rabbit IgG, streptavidin- 344 alkaline phosphatase conjugate, 4-nitro blue tetrazolium chloride, and 5- 289 bromo-4-chloro-3-indolyl phosphate were from Boehringer 378 Mannheim.

Cell Culture—MCF-7/PKC-\(\alpha\) cells were generated by stably co-transfect- 289 ing MCF-7 cells (American Type Culture Collection, Rockville, MD) with 529 PKC-\(\alpha\) subcloned into the pSV-M(2)6 vector and a neomycin re- 417 sistant plasmid as described earlier (19). Cells stably transfected with 450 the empty pSV-M(2)6 vector (MCF-7/vector cells) were also generated 477 and used as one of the control cell lines in this study. MCF-7 cells overexpressing PKC-\(\beta\) were generated by stably transfected MCF-7 487 cells with the complete human PKC-\(\beta\) cDNA (24) subcloned into pRC/ 289 CMV vector (Invitrogen). A corresponding vector control line (express- 487 ing the empty vector) was also developed.\(^a\) The MCF-7/PKC-\(\beta\) cells were 489 selected with G418 as described\(^2\); after selection, these cells contained 498 PKC-\(\beta\) at similar levels as that seen in MCF-7/PKC-\(\alpha\) cells.\(^5\) MCF-7/ 511 PKC-\(\alpha\) cells (suspected), MCF-7/PKC-\(\beta\) cells (attached), and the corre- 514 sponding MCF-7/vector cells (attached) were generated in 75-cm\(^2\) 517 culture flasks (Corning) in Dulbecco's modified Eagle's medium supple- 522 mented with 10% fetal calf serum (National Cancer Institute, NIH, Bethesda, MD), were cultured in NIH 3T3 clone-7 fibroblasts, kindly provided by Dr. Douglas R. Lowy and 535 [\(^{14}C\)]Choline from Prelabeled Phospholipids— 537 the tubes (MCF-7/PKC- 542 absence or presence of PMA and ethanol as indicated. Incubations were 543 performed in quadruplicate, and the experiment in the vector control cells. \\

**Western Blot Analysis of the \(\beta\)-Isomers of PtdEtOH**—MCF-7/PKC-\(\alpha\) cells (5 - 5\(^{10}\) \(\times\) cells) were homogenized with a Dounce homogenizer (100 544 strokes) in 0.5 ml of an extraction buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 25 mg/ml apronin, and 2 mg diethiothreitol. After removing cell debris (by cen- 547 trifugation at 700 \(\times\) g for 6 min), cell lysates were denatured by boiling in Laemmli sample buffer for 5 min. Proteins (50 \(\mu\)g/sample) were 548 separated by electrophoresis on 8% sodium dodecyl sulfate-polyacrylam- 549 ide gels and then transblotted to nitrocellulose membranes. For the detection of immunoreactive proteins, the membranes were first incubated 550 with 2 \(\mu\)g/ml antibodies against the \(\beta\)- and \(\beta\)-PKC isoforms for 2 h, followed by incubations with the second antibody (biotinylated goat 551 anti-rabbit IgG; 1/1000 dilution) for 2 h. Finally, the bands were visu- 552 alized by incubating the membranes first with streptavidin-kaline phospha- 553 tase conjugate for 30 min and then with 4-nitro blue tetrazo- 554 lum chloride and 5-bromo-4-chloro-3-indolyl phosphate. The bands were 555 quantitated as described earlier (27).

**RESULTS**

**Effects of PMA on PLD Activity in MCF-7/PKC-\(\alpha\), MCF- 563 PKC-\(\beta\), and MCF-7/Vector Cells**—We have previously reported 564 that PLD activity in parental MCF-7 cells is negligible (28). Accordingly, in the presence of 150 mM ethanol, MCF-7/vector cells synthesized only a small amount of PtdEtOH, a marker of 566 PLD activity, in response to 100 nM PMA (Fig. 1); this small effect of PMA was not always detectable. PMA also failed to induce significant formation of PtdEtOH in MCF-7/PKC-\(\beta\) cells, or in the corresponding vector control cells (not shown). In contrast, in MCF-7/PKC-\(\alpha\) cells, 100 nM PMA enhanced Pt- 569 dEtOH synthesis about 5-fold in a highly reproducible manner (Fig. 1). In other experiments we determined that maximal stimulation of PtdEtOH synthesis in MCF-7/PKC-\(\alpha\) cells required about 100 nM PMA, while half-maximal stimulation was elicited by about 10 mM concentration of PMA when the incubation time was 30 min (data not shown).

In PMA-stimulated MCF-7/PKC-\(\alpha\) cells the formation of PtdEtOH was the function of ethanol concentration up to 300 mM tested (Fig. 2). Higher than 300 mM concentrations of ethanol had cytotoxic effects (determined by trypan blue exclusion assay); thus, the effects of higher concentrations of ethanol on PtdEtOH formation were not tested. In the absence of eth- 491 anol, PMA enhanced the formation of phosphatidic acid about 2.1-fold (data not shown). At increasing concentrations of eth- 506 anol, PMA failed to enhance, in fact it slightly decreased, the formation of phosphatidic acid, indicating the conversion of PLD activity from the hydrolytic to the transphosphatidylylating function (Fig. 2).

**Fig. 1.** PMA greatly stimulates PtdEtOH formation in MCF-7/ 589 PKC-\(\alpha\) but not in MCF-7/vector cells. MCF-7/vector (I) and MCF-7/ PKC-\(\alpha\) (II) cells were prelabeled with \([^{14}C]\)palmitic acid and then incu- 594 bated with 150 mM ethanol in the absence (\(\square\)) or presence (\(\bigcirc\)) of 100 mM PMA for 30 min as described under “Experimental Procedures.” The data represent the mean of four independent incubations performed on the same day with the same passage of cells. The experimental error in each case was less than 15%. In another experiment (performed in quadruplicate), PMA had similar effects in the overexpressor cells, but it had practically no effect in the vector control cells.

**Fig. 2.**
Previously we found that a multidrug-resistant subline of MCF-7 cells, in contrast to the parental cells, exhibited high levels of PLD activity which preferentially hydrolyzed PtdEtn (28). Thus, increased formation of PtdEtOH in MCF-7/PKC-α cells could reflect increased expression of a PtdEtn-specific PLD. Alternatively, activated PLD in MCF-7/PKC-α cells could act like PLD activity in NIH 3T3 fibroblasts which hydrolyzes both PtdEtn and PtdCho (13). To distinguish between these possibilities, next we separately determined the effects of PMA on PtdEtn and PtdCho hydrolysis using [14C]ethanolamine- and [14C]choline-labeled cells. The incubation condition was such (i.e. 2 mM ethanolamine or 20 mM choline was present) that there was no further metabolism of newly formed [14C]ethanolamine and [14C]choline. As expected, in MCF-7/vector cells PMA elicited only very small increases in the formation of both [14C]ethanolamine (Fig. 3A) and [14C]choline (Fig. 3B) from the respective labeled phospholipids. In five experiments the stimulatory effects of 100 nM PMA on the formation of [14C]ethanolamine and [14C]choline in vector control cells ranged between 1.1–1.35-fold and 1.15–1.6-fold, respectively. Overexpression of PKC-δ failed to detectably enhance the effects of PMA on the hydrolysis of either [14C]PtdEtn (Fig. 3A) or [14C]PtdCho (Fig. 3B). In contrast, in labeled MCF-7/PKC-α cells PMA caused large increases in the hydrolysis of both [14C]PtdEtn (Fig. 3A) and [14C]PtdCho (Fig. 3B). Again, in five experiments performed, the effects of 100 nM PMA on the formation of [14C]ethanolamine and [14C]choline ranged between 2.5–2.9-fold and 5.5–7.2-fold, respectively. It should be added here that the concentration-dependent effects of PMA on the hydrolysis of these two phospholipids were consistently different. Thus, in five experiments half-maximal stimulation of PtdEtn and PtdCho hydrolysis appeared to require 5–10 and 20–25 nM PMA, respectively. Also, maximal stimulation of PtdEtn and PtdCho hydrolysis required about 50 and 100 nM PMA, respectively.

In fibroblasts, maximal inhibition of PLD activity by GF 109203X, an often used selective inhibitor of PKC (29, 30), required around 10 μM concentration of the inhibitor (31). At this concentration GF 109203X inhibited the stimulatory effects of PMA (100 nM) on the hydrolysis of PtdEtn (Fig. 4A) and PtdCho (Fig. 4B) by 73 and 71%, respectively. GF 109203X had no cytotoxic effects during the 40-min incubation period (used here) as determined by the trypan blue exclusion assay. Similarly to GF 109203X, 0.5–3 μM Gö 6976, which in this concentration range selectively inhibits the conventional PKC isozymes (32), significantly inhibited PMA-induced hydrolysis of both PtdEtn (Fig. 5A) and PtdCho (Fig. 5B). Again, Gö 6976 was not cytotoxic during the incubation period used. These data further suggested that PMA-induced hydrolysis of both phospholipids was mediated by a conventional PKC isof orm(s).

One of us recently found that MCF-7/PKC-α cells contain less ε, γ, δ, and μ-PKC enzymes than the wild-type cells. Thus, these PKC isozymes could not possibly mediate the effects of PMA on PtdEtn hydrolysis in MCF-7/PKC-α cells. Similarly, overexpression of PKC-δ failed to enhance the effect of PMA on PLD activity indicating that this isozyme was not a mediator of PMA action either. However, overexpression of PKC-α was also accompanied by increased expression of PKC-β (19). Since these studies were reported (19), we have determined by Western blot analysis that the ratio of PKC-β to PKC-δ in MCF-7/PKC-α cells is about 4:1.3 For this reason, and because only PKC-β (5–7), but not PKC-βII (7), was found to regulate PLD activity in vitro, next we dealt only with the possible regulatory role of PKC-β.

Effects of PMA on PLD Activity in R6 Cells Overexpressing PKC-β, PKC-ε, and PKC-α.—To examine possible regulation of PtdEtn hydrolysis by PKC-β, next we used the previously well characterized R6/PKC-β cells which highly express this isoform (21, 22). Using a 30-min incubation time, in R6/PKC-β and the corresponding vector control cells (which entirely lack PKC-β) the concentration-dependent effects of PMA on PtdEtn hydrolysis were not significantly different (Fig. 6A). However, in agreement with previous observations (5), expression of PKC-β significantly enhanced the stimulatory effect of PMA on PtdCho hydrolysis (Fig. 6B).

Interestingly, overexpression of PKC-α in R6 cells had no major effects on PMA-induced hydrolysis of either PtdEtn (Fig. 6A) or PtdCho (Fig. 6B) when treatments with PMA were performed for 30 min. This could be an indication that the level of PKC-α in these fibroblasts is sufficiently high for optimal regulation of PLD activity by this isozyme at this incubation time. However, if PKC-α is indeed a regulator of PLD, one would expect that overexpression of this isoform will affect the initial activation process, particularly at lower concentrations of PMA. Indeed, when a 10-min incubation time was used, in R6/PKC-α cells lower concentrations (2.5–15 nM) of PMA were about twice more effective in stimulating [14C]PtdEtn hydrolysis than in the corresponding vector control cells (Fig. 7A). At this incubation time (10 min), lower concentrations (2.5–7.5 nM) of PMA were also more effective inducers of [14C]PtdCho hydrolysis in the R6/PKC-α than in the corresponding vector control cells (Fig. 7B). However, higher concentrations (30–100 nM) of PMA had similar effects on [14C]choline formation in the two cell lines. Reduction of incubation time to 10 min did not result in similar relative increases in PMA-induced [14C] PtdEtn hydrolysis in R6/PKC-β cells (data not shown). Reduction of incubation time below 10 min was not feasible because the effects of low concentrations of PMA could not be accurately determined.

3 J. De Vente, K. Ways, W. H. Anderson, and Z. Kiss, unpublished data.
Fig. 3. Concentration-dependent effects of PMA on the hydrolysis of PtdEtn and PtdCho in MCF-7/PKC-α, MCF-7/PKC-θ, and MCF-7/vector cells. MCF-7/vector (empty pSV 2M(2)6 vector) cells (●), MCF-7/PKC-α cells (▲), and MCF-7/PKC-θ cells (■), labeled with [14C]ethanolamine (A) or [14C]choline (B) as described under “Experimental Procedures,” were treated with PMA (0–100 nM) for 30 min. Each point represents the mean ± S.E. of eight incubations performed in two independent experiments. Similar results were obtained in three other experiments each performed in triplicate. In MCF-7 cells expressing the empty pRC/CMV-vector (a corresponding control to MCF-7/PKC-θ cells) PMA also failed to significantly stimulate PtdEtn and PtdCho hydrolysis (four incubations; data are not shown).

In other experiments, overexpression of PKC-ε in Rat-6 fibroblasts failed to enhance the effects of PMA (5–100 nM) on either PtdEtn or PtdCho hydrolysis over a 10–30-min incubation time (data not shown). We also developed an NIH 3T3 cell line overexpressing PKC-ε about 15-fold (33). While this isozyme promoted the stimulatory action of ethanol on PtdEtn hydrolysis (33), it did not alter the effect of PMA on PtdEtn hydrolysis (data not shown).

DISCUSSION

The content of PKC-α in wild-type MCF-7 human breast carcinoma cells is very low (34). Incidentally, in these cell PMA also had only very small effects on the hydrolysis of PtdEtn and PtdCho (28). In contrast, in MCF-7/MDR cells the level of PKC-α is high (34), and this is associated with high expression of a PMA-stimulated PtdEtn-hydrolyzing PLD activity (28). One of the major goals of this work was to determine whether this association between PKC-α and PMA-induced PtdEtn hydrolysis is accidental or causal. If this association is causal, then it should be possible to experimentally induce expression of the PMA-sensitive PtdEtn-hydrolyzing PLD by overexpressing PKC-α in MCF-7 cells. A major finding of this work is that high expression of PKC-α in MCF-7 cells indeed greatly potentiated the effect of PMA on the hydrolysis of PtdEtn (and PtdCho). In addition, overexpression of PKC-α in R6 fibroblasts (which already contains this isozyme at a relatively high level) also enhanced the initial rate of activation of PtdEtn-hydrolyzing PLD by PMA. Finally, Gö 6976, a selective inhibitor of conventional PKC isozymes in the low micromolar concentration range (32), substantially inhibited PMA-induced PLD activity. All these suggest that PKC-α indeed may be a major mediator of PMA effect on PtdEtn hydrolysis. However, MCF-7 cells contain several PKC isozymes, some of them being expressed at different levels upon expression of PKC-α. Thus, another important goal was to determine the possible role of other PKC isozymes in the mediation of PMA effect on PtdEtn hydrolysis.

There are many observations, some of them presented in this work, which indicate that the calcium-independent (novel) isozymes of PKC may not mediate the effects of PMA on PtdEtn hydrolysis. Thus, overexpression of PKC-θ failed to significantly modify the effect of PMA on PtdEtn and PtdCho hydrolysis, indicating that this isozyme is not a regulator of PLD activity. Overexpression of PKC-ε in fibroblasts also had no effect on PMA-induced PtdEtn hydrolysis. In addition, PKC-ε was undetectable both in MCF-7/MDR cells (34) and carcinogen-treated fibroblasts (27), although PMA effectively stimulated PtdEtn hydrolysis in both cases (27, 28). Importantly, high expression of PKC-α in MCF-7 cells actually reduced the cellular levels of the ε, η, γ, and μ-PKC isoforms, which again argues against the mediatory role of these isozymes. Finally, low micromolar concentrations of Gö 6976, which selectively inhibit the conventional (α, β, and γ) but not the novel PKC isoforms (32), also inhibited PMA-induced PtdEtn hydrolysis.

Overexpression of PKC-α in MCF-7 cells also resulted in higher expression of PKC-β1 (19); as mentioned above, most of this increase is attributable to the expression of the β1-isozyme. Taking into account this finding with others indicating that the level of PKC-βII is lower in the MCF-7/MDR cells compared to the parent cells (34), and that PKC-βII is not a regulator of a PtdCho-specific PLD activity in vitro (7), it seems unlikely that PKC-βII is a major mediator of PMA effect on PtdEtn hydrolysis. However, a regulatory role for this isozyme cannot be
entirely ruled out; experiments are underway in this laboratory to clarify this issue.

Overexpression of PKC-βI in Rat-6 fibroblasts failed to enhance the effect of PMA on PtdEtn hydrolysis. Furthermore, MCF-7/MDR cells do not contain detectable amounts of PKC-βI (34), although these cells respond to PMA far better than the parent MCF-7 cells (28). Thus, it seems conceivable that regulation of PtdEtn hydrolysis by PMA does not involve PKC-βI to a significant extent.

PKC-γ could not be detected in any of the cellular systems used here, so obviously this isozyme does not play a role in the regulation of PLD activity.

While PKC-α emerges as a major regulator of PtdEtn hydrolysis, it is presently not clear whether this isozyme has the same dominant role in the regulation of PtdCho hydrolysis in MCF-7/PKC-α cells. For example, fibroblasts contain PKC-α but not PKC-β (27), and in these cells PMA stimulates the hydrolysis of PtdEtn and PtdCho to similar extents (27). In contrast, in MCF-7/MDR cells, PtdCho is a relatively poor substrate of PMA-stimulated PLD (28), despite the high expression level of PKC-α (34). Although overexpression of PKC-α in MCF-7 cells enhanced PMA-induced PtdCho hydrolysis, these cells also contain elevated levels of PKC-β (19), mostly represented by the βI isoform. This is an important consideration, because expression of PKC-βI in R6 fibroblasts resulted in increased PtdCho hydrolysis in the presence of PMA (Ref. 5; Fig. 6B in this work). On the basis of all these observations, it is possible that in the MCF-7/PKC-α cells both PKC-α and PKC-βI contribute to the regulation of PtdCho. The involvement of two PKC isozymes may explain the different concentration dependence of PMA effects on PtdEtn and PtdCho hydrolysis. The less sensitive component of PMA effect on PtdCho hydrolysis may reflect the mediatory role of PKC-βI. This would not be the first example of differential sensitivity of these PKC isozymes to PMA; it has been reported that PKC-βI exhibits somewhat lower affinity to PMA than PKC-α (20).

While it is clear that there are significant differences in the regulation of PtdEtn and PtdCho hydrolysis by the PKC system, PKC-α and PKC-βI may also regulate PtdCho-specific PLD activity by different mechanisms, or they may specifically activate different isofoms of PLD. Otherwise it is difficult to explain how the expression of PKC-βI in R6 cells could enhance the effect of PMA on PtdCho hydrolysis at a 30-min incubation time, when the cellular content of PKC-α appeared to be suffi-
of PKC-α action on PtdEtn hydrolysis as well as the physiological role of this PLD activity remains to be determined.

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