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# Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase C$\gamma$ and protein kinase C$\alpha$ signalling cascade

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Phospholipase D (PLD)1 is quiescent *in vitro* and *in vivo* until stimulated by classical protein kinase C (PKC) isoforms, ADP-ribosylation factor or Rho family members. By contrast, PLD2 has high basal activity, and the mechanisms involved in agonist-induced activation of PLD2 are poorly understood. Using transiently transfected human embryonic kidney (HEK)-293 cells as a model system, we report in the present study that PLD2 overexpressed in HEK-293 cells exhibits regulatory properties similar to PLD1 when stimulated in response to insulin and phorbol ester. Co-expression of PLD1 or PLD2 with PKC$\alpha$ results in constitutive activation of both PLD isoforms, which cannot be further stimulated by insulin. Co-expression of PLD1 with phospholipase C (PLC)$\gamma$ has the same effect, while co-expression of PLD2 with PLC$\gamma$ allows PLD2 activity to be stimulated in an insulin-dependent manner. The PKC-specific inhibitors bisindolylmaleimide and Gö 6976 abolish insulin-induced PLD2 activation in HEK-293 cells co-expressing the insulin receptor, PLC$\gamma$ and PLD2, confirming that not only PLD1, but PLD2 as well, is regulated in a PKC-dependent manner. Finally, we provide evidence that PKC$\alpha$ is constitutively associated with PLD2. In summary, we demonstrate that insulin treatment results in activation of both PLD1 and PLD2 in appropriate cell types when the appropriate upstream intermediate signalling components, i.e. PKC$\alpha$ and PLC$\gamma$, are expressed at sufficient levels.

Key words: regulation, interaction, ADP-ribosylation factor, RhoA.

## INTRODUCTION

A hallmark of mammalian phospholipase D (PLD) is its capacity for activation as a consequence of agonist-induced stimulation of G-protein-coupled and tyrosine kinase receptors (reviewed in [1]). Two PLD isoforms have been cloned and characterized; PLD1 [2] and PLD2 [3,4]. Characterization of recombinant purified human PLD1 has revealed that it is directly activated by classical protein kinase C (PKC) family members and by the small G-proteins ADP-ribosylation factor (ARF) and Rho [5]. Involvement of these signalling molecules in regulating PLD/PLD1 activity in cells has been demonstrated in various studies (reviewed in [1,6,7]), and is further supported by mutant alleles of PLD1, which are selectively unresponsive to PKC, ARF and RhoA [8]. PLD1 has additionally been shown to interact physically with RhoA [9,10], PKC [11–13] and ARF [14]. Whether the PKC and ARF protein–protein interactions are direct or require intermediary proteins or lipids has not been established.

In contrast to PLD1, purified recombinant PLD2 is active and is not further stimulated by addition of PKC$\alpha$, ARF or RhoA [3]. Moreover, PLD2 exhibits high basal activity upon overexpression in COS-7 cells, under conditions where PLD1 is quiescent until stimulated by agonists, such as phorbol esters (which activate PKC) or activated RhoA. These findings led to the initial conclusion that PLD2 is constitutively active and regulated primarily by inhibitory mechanisms [3,16]. More recent data, however, suggest at least partially overlapping regulatory mechanisms for PLD1 and PLD2. PLD2 overexpressed in COS-7 or SF9 cells can be stimulated (30–50%) *in vitro* by ARF [13,17]. This stimulation by ARF is far more pronounced (13-fold) when the high basal activity of PLD2 is reduced through removal of the N-terminal 308 amino acids [13,17,18]. PLD2 also exhibits a modest response (2-fold increase in activity) to phorbol ester [3], suggesting a positive role for PKC isoforms in the regulation of PLD2 activity in cells. In addition, we have reported that both PLD1 and PLD2 can be activated in HEK-293 cells upon epidermal growth factor (EGF) receptor stimulation [19]. Finally, insulin-stimulated recruitment of the Raf-1 kinase to the plasma-membrane involves PLD2 in HIRcB fibroblasts (Rat-1 fibroblasts overexpressing the human insulin receptor). A catalytically inactive allele of PLD2 appears to act as a dominant-negative in this pathway, blocking Raf-1 translocation to the plasma-membrane unless the inhibition is overcome through the exogenous addition of phosphatidic acid, the lipid product generated by PLD [20]. Whether insulin-induced stimulation of PLD2 activity is essential for Raf-1 translocation in these cells has not yet been determined. Recently, it was shown that endogenous PLD in HEK-293 cells is activated in response to insulin stimulation. The activation is regulated by the concerted action of PKC and RalA [21], and ARF [22,23]. However, these studies did not determine which PLD isoform was activated by insulin or which signalling proteins linked the insulin receptor to the activation of PLD1 and/or PLD2.

Using transiently transfected HEK-293 fibroblasts as a model system, we report in the present study that both PKC$\alpha$ and PLC$\gamma$ are involved in insulin-induced PLD1 and PLD2 activation.

Abbreviations used: ARF, ADP-ribosylation factor; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HEK, human embryonic kidney; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol.

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Furthermore, similarly to PLD1, PLD2 also associates with PKCα.

## EXPERIMENTAL

### cDNAs

pCGN expression vectors containing the cDNAs of wild-type and inactive human PLD1, wild-type and inactive mouse PLD2, and plasmid pEF-BOS-HA-*rhoA*$^{Val\text{-}14}$ have been described previously [2,3,8,9]. The inactive PLD1 (K898R) and PLD2 (K758R) alleles do not act as dominant-negatives in transient transfection assays that measure PLD activity and were used as negative controls. The empty expression vector (pCGN) was additionally used as a negative control in some experiments. pCGN encodes an influenza-epitope (haemagglutinin; HA) tag that becomes expressed in frame at the N-terminus of the PLDs. The cDNAs for the human insulin receptor type A, PKCα and PLCγ1 were also cloned into a cytomegalovirus promotor-enhancer-driven expression vector.

### Cell line

HEK-293 cells from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, 10% (v/v) fetal calf serum, 2 mM L-glutamine, 10 mg/ml streptomycin and 100 units/ml penicillin (Life Technologies, Tåstrup, Denmark) at 37 °C in a 5% $CO_2$-enriched, humidified atmosphere. The cells were starved in DMEM containing high glucose (25 mM), 0.5% fetal calf serum, 2 mM L-glutamine, 10 mg/ml streptomycin and 100 units/ml penicillin overnight prior to insulin (160 nM) stimulation for 30 min.

### Transfection and transient expression

Transient transfection of HEK-293 cells was performed essentially as described by Chen and Okayama [25], and Gorman et al. [26], or as described previously [3,19]. Confluent HEK-293 cells were treated with trypsin and re-plated in an appropriately sized dish. The cells were diluted to 25% confluency in DMEM containing high glucose, 10% fetal calf serum, 2 mM L-glutamine, 10 mg/ml streptomycin and 100 units/ml penicillin. The following day the cells were transfected. For PLD assays, in 2-cm dishes, 0.5 μg of cDNA was transfected by mixing with 50 μl of 0.2 M $CaCl_2$, adding 50 μl of 50 mM Bes (pH 6.95)/280 mM NaCl/1.5 mM $Na_2HPO_4$ and incubating for 15 min at room temperature before adding the mixture to the cells. For immunoprecipitations, in 3-cm dishes, 7 μg of DNA was transfected per dish by mixing with 75 μl of 0.2 M $CaCl_2$ and 75 μl of 50 mM Bes (pH 6.95)/280 mM NaCl/1.5 mM $Na_2HPO_4$, and incubating for 15 min at room temperature before adding the mixture to the cells. The cells were incubated in an humidified incubator containing 3% $CO_2$ overnight at 37 °C. The next morning the medium was changed and the cells were incubated in an humidified incubator containing 5% $CO_2$. In the evening the cells were starved with the appropriate medium and the experiments were performed the following day.

### Antibodies

Anti-phosphotyrosine (PY20) antibody was obtained from Transduction Laboratories. Anti-(HA-tag) monoclonal antibody was obtained from Boehringer Mannheim. Rabbit polyclonal antisera were generated in rabbits immunized with peptides covering the C-terminal 15 amino acids of PKCα and the C-terminal 15 amino acids of the insulin receptor. PLCγ polyclonal rabbit antiserum was provided by Dr. A. Ullrich, Max-Planck-Institut für Biochemie, Martinsried, Germany [27].

### Cell lysis, immunoprecipitations and gel electrophoresis

The cells were transfected with 0.4 μg of human insulin receptor cDNA, 1.2 μg of any other given cDNA and empty vector was added to a final concentration of 7 μg of DNA as described above to keep the total amount of DNA transfected constant. Prior to lysis, the cells were treated with insulin (160 ng/ml) for 30 min and washed once with PBS. Cells were lysed with 0.4 ml of ice-cold lysis-buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 20 mM NaF, 200 μM $Na_3VO_4$, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF; Calbiochem) and 1 mg/ml *p*-nitrophenyl phosphate (Sigma)] per 3-cm dish and precleared by centrifugation at 15000 ***g*** for 10 min at 4 °C. Immunoprecipitations were performed by addition of 30 μl of Protein G–Agarose (Boehringer-Mannheim) and anti-(HA-tag) monoclonal antibody for 3 h, followed by brief centrifugation. The precipitates were washed three times in washing-buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Nonidet P40, 10% glycerol, 20 mM NaF, 200 μM $Na_3VO_4$, 1 mM AEBSF and 1 mg/ml *p*-nitrophenyl phosphate]. For gel electrophoresis the immunoprecipitates were dissolved in 2 × Laemmli buffer, and the proteins were separated by SDS/PAGE and transferred on to nitrocellulose membranes (BA85; Schleicher and Schuell, Dassel, Germany). The immunoreactive proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham).

### PLD assay in intact cells

*In vivo* measurements of PLD activity were performed as described previously [9,19]. For experiments involving insulin stimulation, the cells were transfected with the given combination of cDNA; 0.02 μg of human insulin receptor cDNA, 0.06 μg of any other given cDNA, and empty vector was added to a final concentration of 0.5 μg cDNA as described above to keep the total amount of DNA transfected constant. The cells were labelled with 4 μCi of [$^{14}$C]palmitic acid per 2-cm dish, added to the starvation medium and incubated overnight at 37 °C in a 5% $CO_2$-enriched, humidified atmosphere. The cells were washed twice in HHBG buffer [10 mM Hepes (pH 7.4), 1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.37 mM KCl, 137 mM NaCl, 4.2 mM $NaH_2PO_4$, 1% (w/v) BSA and 10 mM glucose] and incubated in 0.3% butan-1-ol in HHBG buffer for 20 min at 37 °C. Subsequently, the cells were treated with or without insulin (160 ng/ml) for 30 min. After incubation the buffer was aspirated and 0.5 ml of ice-cold methanol was added to each dish. The cell debris was scraped into a glass vial and kept on ice. Chloroform and $H_2O$ were added to give a final chloroform/methanol/$H_2O$ ratio of 1:1:0.8 (by vol.). After vortex-mixing, the vials were centrifuged at 1200 ***g*** for 5 min and the organic phase was spotted on to Whatman LK5DF TLC plates. The labelled products were separated by TLC using the upper phase of a mixture of 2,2,4-trimethylpentane/ethylacetate/acetic acid/water ratio of 5:11:2:10 (by vol.). PhosphorImager technology (Molecular Dynamics, Little Chalfont, Bucks., U.K.) was used to determine the amount of labelled phosphatidylbutanol (PtdBut) and the amount of total lipid. In each experiment the PLD activity was determined in triplicate; the average values are shown in the Figures together with S.E.M.s, represented by error bars. A representative of each experiment is given. The expression levels of given proteins were measured by Western blot analysis.

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For inhibitor studies, PLD activity was measured after a 40 min preincubation with 1 $\mu$M Gö 6850 or 1 $\mu$M Gö 6976 dissolved in DMSO (Calbiochem). The inhibitors were also present during the insulin stimulation, and control cells were exposed to DMSO alone.

### *In vitro* PLD assay

PLD activity assays were carried out using the head group release assay for a 30 min time period as described previously (method described in [28]; see [2,3,5,8,9,13,18] for additional data using this assay system). Recombinant ARF1, *rhoA*, and PKC$\alpha$ were purified and activated using either 50 $\mu$M GTP[S] or 100 nM PMA as previously described [5,29]. All results shown used activated ARF, Rho or PKC$\alpha$. The activation of PLD by small G-proteins is strictly GTP-dependent; activation by PKC is strongly stimulated in the presence of PMA, but is ATP-independent. [$^3$H]Dipalmitoyl phosphatidylcholine was obtained from NEN Life Science Products (Boston, MA, U.S.A.). GTP[S] was purchased from Roche (Indianapolis, IN, U.S.A.), PMA from Sigma (St. Louis, MO, U.S.A.) and lipids from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All other reagents were of analytical grade, unless otherwise specified.

## RESULTS

### PKC$\alpha$-induced PLD1 and PLD2 activation

To begin to establish the relationship between insulin signalling and PLD stimulation, we examined their activity upon over-expression, as well as upon co-transfection with known PLD activators. We previously reported that although PLD2 exhibits unregulated activity *in vitro* in the purified state and *in vivo* after overexpression in COS-7 cells [3], it nonetheless can be stimulated by EGF in HEK-293 cells [19]. This prompted us to re-address the regulation of PLD2 in HEK-93 cells (Figure 1). PLD2, PLD1 and a catalytically-inactive allele of PLD1 (K898R, see [9]) were transfected into HEK-293 cells, harvested and assayed *in vitro* in the basal state, as well as in the presence of the PLD1 stimulators, ARF1, RhoA and PKC$\alpha$ (Figure 1, left panel). As reported previously for COS-7 cells [3], HEK-293 cells also exhibited relatively low levels of endogenous PLD activity, as indicated by the control cells that were transfected with catalytically inactive PLD1 (K898R). Consistent with previous reports, wild-type overexpressed PLD1 exhibited very low basal activity under these conditions and was stimulated robustly (8–21-fold) by ARF1, RhoA and PKC$\alpha$. PLD2, as reported previously for COS-7 cells, exhibited significantly elevated basal *in vitro* activity, which was further increased by ARF1 (less than 2-fold), while the addition of RhoA had no effect. However, in contrast to a prior report [3], PLD2 exhibited a significant response to PKC$\alpha$, which was greater than 3-fold (Figure 1, left panel). Extension of these studies, measuring PLD activity in intact HEK-293 cells as the accumulation of PtdBut (Figure 1, right panel), revealed an even more dramatic enhancement of PLD activity by PMA. As anticipated [8], the endogenous response [pCGN (empty vector) or K898R-transfected cells] to co-transfected activated Rho (RhoA$^{Val-14}$) or to PMA (a potent PKC activator) was absent or modest respectively. Transfected wild-type PLD1 exhibited a low basal activity, which was stimulated by co-transfection of activated RhoA or upon PMA treatment. PLD2 overexpressed in HEK-293 fibroblasts exhibited relatively low basal activity, relative to its reported high basal activity in COS-7 cells [3,13], and manifested a very strong response to the PKC-activating agent PMA. In contrast to PLD1, however, PLD2 appeared to be insensitive to activated RhoA (Figure 1, right panel). The *in vitro* and cell-based data suggest common, but also specific mechanisms for the activation of PLD1 and PLD2. Although Rho and PKC [30] translocate to membrane surfaces once activated, this is unlikely to represent the mechanism that regulates PLD, since PLD1 and PLD2 are constitutively membrane-associated [7]. The findings also indicate that over-expressed PLD2 in HEK-293 cells, as opposed to COS-7 cells, is sufficiently regulated to allow investigations that are aimed at identifying signalling proteins involved in the regulation of PLD activity in response to insulin.

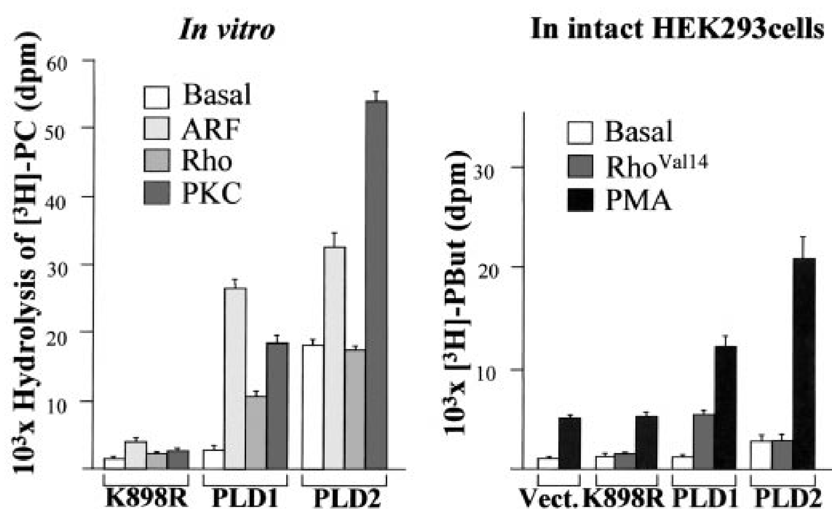


**Figure 1 PLD2 overexpressed in HEK-293 cells exhibits a response to PKC$\alpha$ *in vitro* and to PMA *in vivo***

Left panel: lysates from HEK-293 cells transiently transfected with wild-type PLD1, wild-type PLD2, and catalytically-inactive PLD1 were assayed *in vitro* for basal and effector-stimulated responses as described in the Experimental and Results sections. Right panel: cells transfected as above or with the empty vector, pCGN (Vect.), were assayed in cells for basal, PMA-stimulated, and co-transfected Rho$^{Val-14}$-stimulated PLD activity. The data are means ± S.E.M. of one experiment performed in triplicate. The results shown are representative of at least three independent experiments.

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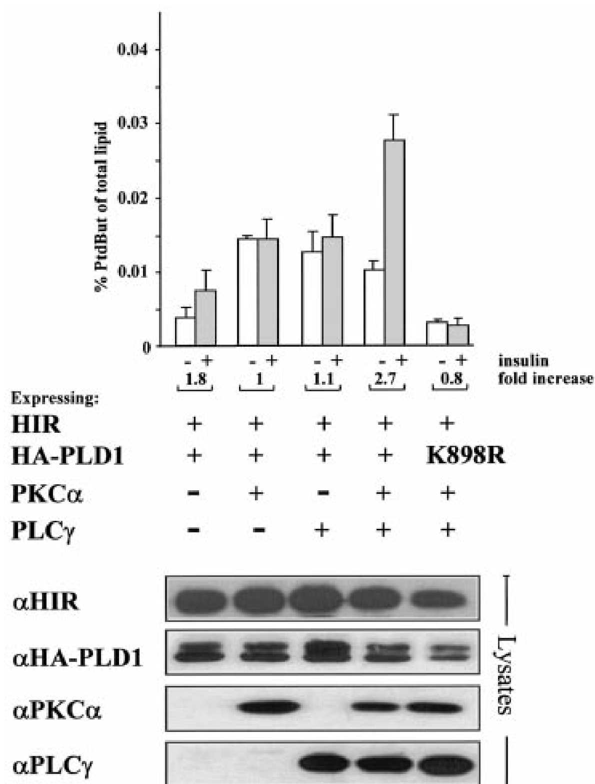


**Figure 2 The activation of PLD1 in response to insulin is regulated by PKC$\alpha$ and PLC$\gamma$**

HEK-293 cells were transiently transfected with the indicated combinations of the human insulin receptor (HIR), PKC$\alpha$, PLC$\gamma$ and wild-type or catalytically inactive (K898R) HA-PLD1. PLD activity was determined by measuring the accumulation of PtdBut relative to total lipids in unstimulated cells (−, white bars), and cells treated for 30 min with insulin (+, grey bars) as described in the Experimental section. The data are means ± S.E.M. of one experiment performed in triplicate. The level of expression of the indicated genes was determined by Western blot analysis of total cell lysates using anti-HIR ($\alpha$HIR), anti-PKC$\alpha$ ($\alpha$PKC$\alpha$), anti-PLC$\gamma$ ($\alpha$PLC$\gamma$) and anti-HA ($\alpha$HA) specific antibodies (lower panels). The immunoreactive proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and the ECL® detection method. The result shown is representative of at least three independent experiments.

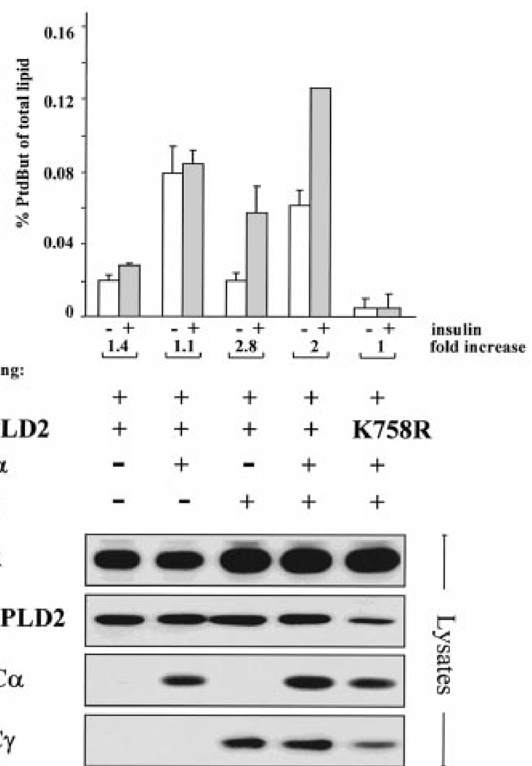


**Figure 3 Insulin-induced activation of PLD2 in response to insulin requires PLC$\gamma$ and PKC$\alpha$**

HEK-293 cells were transiently transfected with the indicated combinations of the human insulin receptor (HIR), PKC$\alpha$, PLC$\gamma$ and wild-type or catalytically inactive (K758R) HA-PLD2. PLD activity was determined by measuring the accumulation of PtdBut relative to total lipids in unstimulated cells (−, white bars), and cells treated for 30 min with insulin (+, grey bars) as described in the Experimental section. The data are means ± S.E.M. of one experiment performed in triplicate. The level of expression of the indicated genes was determined by Western blot analysis of total cell lysates using anti-HIR ($\alpha$HIR), anti-PKC$\alpha$ ($\alpha$PKC$\alpha$), anti-PLC$\gamma$ ($\alpha$PLC$\gamma$) and anti-HA ($\alpha$HA) specific antibodies (lower panels). The immunoreactive proteins were visualized using horseradish peroxidase-coupled secondary antibodies and the ECL® detection method. The result shown is representative of at least three independent experiments.

### Insulin-induced activation of PLD1 and PLD2

Insulin-induced activation of PLD in HIRcB cells was previously shown to be ARF dependent [22], and in rat adipocytes it was shown to be Rho- and ARF-dependent [24]. In HEK-293 cells the endogenous PLD activity was shown to be dependent on RalA and PKC [21]. These investigations, however, did not identify the PLD isoforms involved. We therefore first set out to establish an overexpression system in which specific PLD isoform activation in response to insulin stimulation could be examined and to determine which signalling proteins are involved in regulating this response pathway. PLD activity was measured in transiently transfected HEK-293 fibroblasts as the accumulation of PtdBut in untreated and insulin-stimulated cells. A time-course analysis revealed that PLD was activated rapidly and remained active for at least 30 min (results not shown). Accordingly, all PLD activity measurements were conducted using a 30 min period of stimulation. Transfection of the human insulin receptor alone did not lead to any induction of PLD activity after insulin stimulation (results not shown). This level of PtdBut was considered as background PLD activity and was subtracted from all shown PtdBut measurements. Co-transfection of the human insulin receptor and either PLD1 (Figure 2) or PLD2 (Figure 3) led to increases in PLD activity (1.4–1.8-fold), indicating that both PLD1 and PLD2 are modestly insulin responsive under these conditions.

Because endogenous PLD activation, in response to other hormones, has been shown to require PKC$\alpha$ and PLC activity [21,31–34], we next examined whether co-transfection of the insulin receptor with PKC$\alpha$ and/or PLC$\gamma$ would lead to further insulin-induced activation of PLD1 and PLD2. As shown in Figures 2 and 3, co-expression of the human insulin receptor, PKC$\alpha$ and either PLD1 or PLD2 resulted in increased basal PLD activity, which was not further stimulated by insulin treatment (Figures 2 and 3). Co-expression of the human insulin receptor, PLC$\gamma$ and PLD1 again raised the basal PLD1 activity and insulin was not able to further stimulate the activity (Figure 2). In contrast, co-transfection of the human insulin receptor, PLC$\gamma$ and PLD2 had no effect on the basal PLD2 activity and insulin treatment stimulated the activity 2.8-fold (Figure 3). Co-transfection of all four proteins (human insulin receptor, PKC$\alpha$, PLC$\gamma$ and PLD1, or human insulin receptor, PKC$\alpha$, PLC$\gamma$ and

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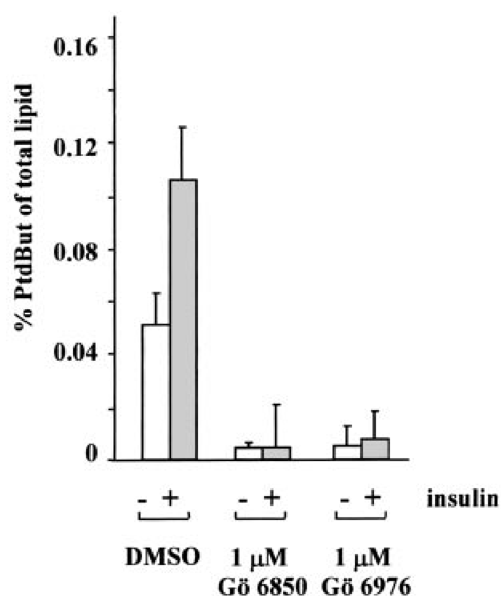


**Figure 4 PKC inhibitors abolish insulin-induced PLD2 activation**

HEK-293 cells were transiently transfected with the human insulin receptor (HIR), HA-PLD2 and PLC$\gamma$. The cells were treated with DMSO, Gö 6850 (1 $\mu$M) or Gö 6976 (1 $\mu$M) for 1 h prior to insulin-induced PLD2 activation, which was determined by measuring the accumulation of PtdBut relative to total lipids in unstimulated cells (—, white bars), and cells treated for 30 min with insulin (+, grey bars) as described in the Experimental section. The data are means ± S.E.M. of one experiment performed in triplicate. The result shown is representative of two independent experiments.

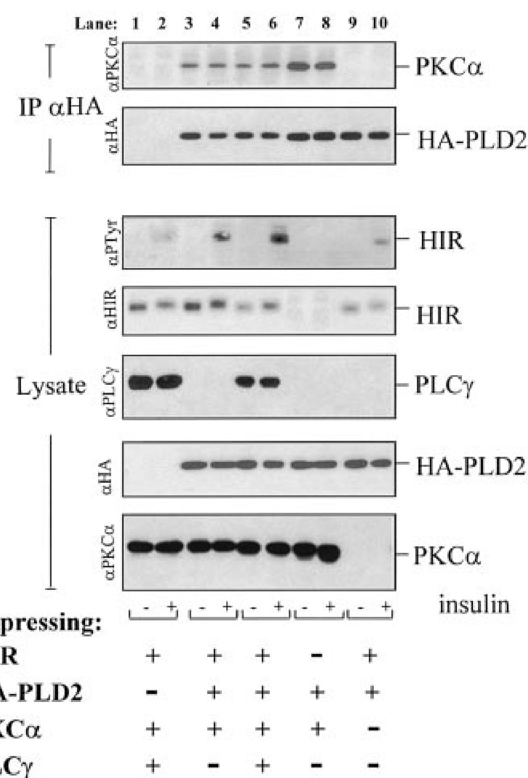


**Figure 5 PLD2 forms a physical complex with PKC$\alpha$**

HEK-293 cells were transiently transfected with: the human insulin receptor (HIR), PKC$\alpha$ and PLC$\gamma$ (lanes 1 and 2); HIR, HA-PLD2 and PKC$\alpha$ (lanes 3 and 4); HIR, HA-PLD2, PKC$\alpha$ and PLC$\gamma$ (lanes 5 and 6); HA-PLD2 and PKC$\alpha$ (lanes 7 and 8); HIR and HA-PLD2 (lanes 9 and 10) expression plasmids. The cells were either left untreated or were stimulated with insulin for 30 min and subsequently lysed. The HA-PLD2 was immunoprecipitated (IP $\alpha$HA) from all samples using anti-HA antibodies and the proteins were separated by SDS/PAGE. Co-immunoprecipitation of PKC$\alpha$ with HA-PLD2 was determined by Western blot analysis using anti-PKC$\alpha$ specific antibodies (PKC$\alpha$, top panel) and the immunoprecipitation of PLD2 was confirmed using anti-HA antibodies (second panel). Insulin-induced tyrosine phosphorylation of the insulin receptor $\beta$-subunit, as well as the expression of HIR, PLC$\gamma$, HA-PLD2 and PKC$\alpha$, was determined in total cell extracts (Lysate) with phosphotyrosine-specific antibody PY20 ($\alpha$Ptyr), anti-HIR ($\alpha$HIR), anti-PLC$\gamma$ ($\alpha$PLC$\gamma$), anti-HA ($\alpha$HA) and anti-PKC$\alpha$ ($\alpha$PKC$\alpha$) specific antibodies (lower panels). Proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and the ECL® detection method. The result shown is representative of at least three independent experiments.

PLD2) raised the basal activity and, importantly, insulin stimulated the activity of both PLD1 and PLD2, by 2.7- and 2-fold respectively (Figures 2 and 3). To demonstrate that the PLD activity measured in these experiments was indeed mediated by the exogenous PLD isoforms, we included catalytically inactive forms of either PLD1 (K898R) or PLD2 (K758R). As shown in Figures 2 and 3, co-transfection of the catalytically inactive forms of either PLD1 or PLD2 with the human insulin receptor, PKC$\alpha$ and PLC$\gamma$ resulted in the detection of only very minor PLD activity, which was unresponsive to insulin stimulation. The reproducibility of the experiment was tested; and in six experiments the insulin-induced induction of PLD1 activity in human insulin receptor-, PKC$\alpha$-, PLC$\gamma$- and PLD1-transfected cells varied from 2.7–7.2-fold, giving an average of 4.8 with an S.E.M. of 1.8. In eight experiments the insulin-induced induction of PLD2 activity in human insulin receptor-, PKC$\alpha$-, PLC$\gamma$- and PLD2-transfected cells varied from 1.7–2.5-fold, giving an average of 2.0 and an S.E.M. of 0.3. We concluded from these experiments that PKC$\alpha$ and PLC$\gamma$ were involved in the regulation of both PLD1 and PLD2. The results suggest that the endogenous level of PKC$\alpha$ was sufficient to stimulate HA-PLD2, when PLC$\gamma$ was co-transfected with HA-PLD2 and the human insulin receptor. In order to confirm the involvement of endogenous PKC isoforms in insulin-induced PLD2 activation we employed two PKC inhibitors, Gö 6850 and Gö 6976. As shown in Figure 4, incubation of human insulin receptor-, PLD2- and PLC$\gamma$-transfected cells with 1 $\mu$M Gö 6850 or 1 $\mu$M Gö 6976 for 1 h prior to insulin stimulation reduced the basal PLD2 activity and fully abolished its responsiveness to insulin. These data further support the finding that the basal activity, as well as the insulin-induced PLD2 activity, is regulated in a PKC-dependent manner.

### PLD2 forms a physical complex with PKC$\alpha$

Complex formation between PLD1 and PKC$\alpha$ is well documented and the interaction sites of PLD1 have been suggested to be an N-terminal one that interacts with the PKC$\alpha$ regulatory domain and a central or C-terminal one that interacts with the PKC$\alpha$ catalytic domain [8,18,35]. Since our data clearly indicate that the basal as well as the insulin-induced activation of not only PLD1 but also PLD2 requires PKC$\alpha$, we next examined whether PLD2 could form a physical complex with PKC$\alpha$. Complex formation was analysed by Western blot analysis after immunoprecipitation of PLD2 from cells expressing: human insulin receptor, PKC$\alpha$ and PLC$\gamma$ (Figure 5, lanes 1 and 2); human insulin receptor, PKC$\alpha$ and PLD2 (lanes 3 and 4); human insulin receptor, PKC$\alpha$, PLC$\gamma$ and PLD2 (lanes 5 and 6); PKC$\alpha$ and PLD2 (lanes 7 and 8); and human insulin receptor and PLD2 (lanes 9 and 10). Immunoprecipitation of PLD2 resulted in co-immunoprecipitation of PKC$\alpha$ whenever both

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proteins were co-expressed (Figure 5, upper panel, lanes 3–8). The amount of PKC$\alpha$ which co-immunoprecipitated with HA-PLD2 was similar in untreated and insulin-treated cells, and required neither the presence of the insulin receptor (lanes 7 and 8) nor the presence of PLC$\gamma$ (lanes 3 and 4 and 7 and 8). These data indicated that PLD2 forms a physical complex with PKC$\alpha$, and this may be important for the regulation of PLD2 activity.

## DISCUSSION

It has been assumed that the regulation of PLD1 and PLD2 occurs in an isoform-specific manner. PLD1 was shown to have low basal activity and to be regulated *in vitro* by PKC$\alpha$, ARF and Rho. PLD2 meanwhile was reported to exhibit high basal activity under *in vitro* conditions, as well as upon overexpression in COS-7 cells. Therefore it was generally assumed that PLD2 activity was primarily regulated by specific inhibitory proteins in cells [3,16]. Not only the activity, but also the subcellular localization of PLD1 and PLD2 appears to be distinct. While overexpressed PLD1 is localized to membrane vesicles in perinuclear regions in rat embryo fibroblasts, overexpressed PLD2 is localized primarily to the plasma-membrane [3]. Furthermore, PLD2, but not PLD1, forms a physical complex with the EGF receptor and becomes tyrosine phosphorylated in response to EGF stimulation [19].

Co-transfection of the human insulin receptor and either PLD1 or PLD2 in HEK-293 fibroblasts leads to a small increase in insulin-induced PLD activity (1.8- and 1.4- fold respectively), showing that both PLD isoforms respond to agonist stimulation. Additional co-expression of PLC$\gamma$ leads to a marked insulin-induced activation (2.8-fold) of PLD2, while the activity of PLD1 is elevated in the basal state and not further stimulated by insulin. Co-expression of the insulin receptor, PLD1 or PLD2, and PKC$\alpha$ results in elevated basal activity of PLD1 and PLD2 activity, which is unresponsive to insulin treatment in both cases. Co-expression of the insulin receptor and PLD1 or PLD2 together with PLC$\gamma$ and PKC$\alpha$ again results in elevated basal PLD activity, which is, however, further responsive to insulin stimulation (2.7-fold for PLD1, and 2-fold for PLD2). These data show that overexpression of PLC$\gamma$ is required and sufficient to allow potent insulin-induced activation of PLD2 under the conditions used, while the insulin-induced activation of PLD1 requires the overexpression of both PLC$\gamma$ and PKC$\alpha$. Treatment of HEK-293 cells overexpressing the insulin receptor, PLC$\gamma$ and PLD2, with the PKC inhibitors Gö 6850 and Gö 6976 clearly indicates that the activation of PLD2 in response to insulin is PKC-dependent. These data show that the insulin-induced activation of both PLD1 and PLD2 involves PLC$\gamma$ and PKC$\alpha$. However, while the endogenous PKC level appears to be sufficient to mediate activation of PLD2, PKC$\alpha$ overexpression is required to allow activation of PLD1 in response to insulin treatment. Therefore PLD1 and PLD2 differ in their sensitivity to PKC$\alpha$ levels, suggesting distinct mechanisms of activation. One obvious possibility is that PKC stimulates PLD1 through multiple mechanisms. Non-phosphorylation-dependent stimulation of PLD1 by interaction with the PKC regulatory domain has been demonstrated [5], and phosphorylation of unknown effectors and/or possibly PLD1 itself has been suggested [35].

Overexpression of PLC$\gamma$ leads to restoration of the insulin-signalling cascade leading to activation of PLD2, and elevates the basal activity of PLD1. The elevated activity of PLD1 could be caused by constitutive activation of PKC$\alpha$, which is activated by the overexpression of PLC$\gamma$. However, constitutively active PKC$\alpha$ leads to down-regulation of the human insulin receptor [27], which we do not observe. Therefore it is unlikely that overexpression of PLC$\gamma$ leads to constitutive activation of PLC$\gamma$ and thereby constitutively activated PKC$\alpha$ under these conditions. The requirement for PLC$\gamma$ is reasonable, since diacylglycerol (DAG), which can be generated by PLC-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$), activates PKC$\alpha$.

Several studies have shown that PLC$\gamma$ plays a role in the regulation of endogenous PLD. The activation of PLC$\gamma$ is necessary for stimulation of PLD by platelet-derived growth factor [33], and stimulation of PLD in response to EGF requires the activity of PKC and PLC in Swiss 3T3 cells [34]. PLC$\gamma$2 is essential for B cell receptor-induced PLD activation [31]. In CHO-CCK-A cells (Chinese-hamster ovary cells expressing the cholecystokinin-A receptor), receptor-mediated PLD activation is completely dependent on PKC, and the extent to which PLD becomes activated depends largely, if not entirely, on the magnitude and duration of the agonist-induced increase in intracellular $Ca^{2+}$ [31], which together with DAG is required to fully activate PKC$\alpha$. The mechanism of insulin-induced activation of PLC$\gamma$/PKC remains poorly understood. PLC$\gamma$ may be activated in response to insulin-induced stimulation, at least in some tissues. In 3T3-L1 adipocytes, PLC$\gamma$ has been shown to be involved in the metabolic and possibly the mitogenic effect of insulin [36], which suggests that PKC may be activated by PLC$\gamma$-mediated hydrolysis of $PIP_2$ to DAG and inositol 1,4,5-trisphosphate. Our data in the present study indeed suggest that PLC$\gamma$ is involved in insulin signal-transduction leading to the activation of PLD1/PLD2 when PLC$\gamma$ is present at adequate levels.

Complex formation between PLD1 and PKC$\alpha$ is well documented, and the PLD1 interaction sites have been suggested to be an N-terminal site that interacts with the PKC$\alpha$ regulatory domain and a central or C-terminal site that interacts with the PKC$\alpha$ catalytic domain [3,8,18]. Furthermore, PKC$\alpha$ translocates to complex with the human insulin receptor upon insulin stimulation [30]. We have shown in the present study that immunoprecipitation of PLD2 results in co-immunoprecipitation of PKC$\alpha$, suggesting that the proteins are physically associated *in vivo*. However, in contrast to the PLD1–PKC$\alpha$ complex, which is PMA-induced [12], the complex formation of PLD2 and PKC$\alpha$ occurs independently of agonist stimulation. Activation of PKC$\alpha$ in this setting may lead to phosphorylation-dependent conformational changes in PLD2 that alter its activity. Alternatively, activation of PKC may lead to an altered interaction of PLD2 with the membrane, resulting in more effective access to its substrate or to its required lipid co-factor, $PIP_2$. PLD2 and PKC$\alpha$ are both present in caveoli and the plasma-membrane [37,38] and could be associated through the formation of a multiprotein complex, as has been suggested for PLD2 in other contexts [39]. Finally, PKC could act to oppose inhibitors of PLD2 activity found in HEK-293 cells, but not in COS-7 cells, if PLD2 inhibitors are present in HEK-293 and underlie the intrinsic differences in PLD2 basal activities in the two cell lines. Many of the reported PLD inhibitors bind $PIP_2$ in a regulated manner and could accordingly limit the access of both PLD1 and PLD2 to their required lipid co-factors, independent of PKC interaction with PLD if PKC activation regulates the inhibitors themselves.

It has been suggested that PLD2 activity is regulated in an ARF-dependent manner in cells [18], and that the endogenous PLD response to insulin requires ARF, but not Rho [22]. Our results are not in conflict with these data and together they suggest that an ARF- and PLC$\gamma$/PKC$\alpha$-dependent mechanism could act synergistically, similarly to the synergistic activation of PLD1 activity *in vitro* promoted by ARF, PKC$\alpha$ and Rho [5,8].

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In summary, we show that both PLD1 and PLD2 are activated in response to insulin treatment and that their activation involves PKC$\alpha$ and PLC$\gamma$. Further investigations are required to fully understand the mechanism of activation involved and the roles PLD1 and PLD2 play in the biological processes triggered by insulin signalling.

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