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Type Iα phosphatidylinositol 4-phosphate 5-kinase is a putative target for increased intracellular phosphatidic acid

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Abstract Despite the fact that phosphatidic acid (PtdOH) has been implicated as a lipid second messenger for nearly a decade, its intracellular targets have remained unclear. We sought to investigate how an increase in the level of PtdOH could modulate phosphatidylinositol 4-phosphate 5-kinase (PIPkin), an enzyme involved in phosphatidylinositol 4,5-bisphosphate synthesis. Transfection of porcine aortic endothelial (PAE) cells with haemagglutinin (HA)-tagged type Iα PIPkin followed by immunofluorescence confocal microscopy revealed the enzyme to be localised to the plasma membrane. When the transfected PAE cells were stimulated with lyso-PtdOH, increased PIPkin activity was found to be associated with HA immunoprecipitates in an in vitro assay. This PIPkin activation was found to be greatly reduced by prior treatment of the cells with 1-butanol, thereby implicating phospholipase D (PLD) as the in vivo generator of PtdOH. In order to determine if the PtdOH-dependent activation of type Iα PIPkin was dictated by a specific molecular composition of PtdOH, the wild type murine and porcine α isoforms of diacylglycerol kinase (DGK) were individually co-transfected along with type Iα PIPkin. Under these conditions an increase in type Iα PIPkin lipid kinase activity was found in HA immunoprecipitates in an in vitro assay. No increases in lipid kinase activity were observed when type Iα PIPkin was co-transfected with either the human DGKε isoform or a kinase-dead mutant of the murine DGKα isoform. These results provide the first direct evidence for the unification of the production of saturated/monounsaturated PtdOH (through two different routes, PLD and DGK) and the in vivo activation of type Iα PIPkin by this lipid second messenger.

Key words: Phosphatidic acid; 5-Kinase; Phosphatidylinositol 4-phosphate 5-kinase; Lipid second messenger; Signal transduction

1. Introduction

The regulation of the phosphoinositide family of phospholipids requires the balance of phosphoinositide kinases, phosphoinositide phosphatases and phospholipases. Phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) is a phosphoinositide which plays a pivotal role in signal transduction mechanisms. It may be hydrolysed by phosphatidylinositol-specific phospholipase C generating the classical second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [1] or it may be phosphorylated by phosphatidylinositol 3-kinase, to form the more novel lipid second messenger phosphatidylinositol 3,4,5-trisphosphate and other 3-phosphorylated phosphoinositides [2]. In addition to PtdIns4,5P_2 being a substrate, it is also a cofactor for a variety of enzymes and plays roles in intracellular trafficking and reorganisation of the cytoskeleton [3]. Up to recently, the only route of PtdIns4,5P_2 synthesis known was through the sequential phosphorylation of phosphatidylinositol, the last step being catalysed by a 5-kinase. This 4-phosphate 5-kinase reaction is now known to be catalysed by the type I family of the phosphatidylinositol 4-phosphate 5-kinase (PIPkin) superfamily and probably accounts for over 95% of the total PtdIns4,5P_2 synthesis. The type I family consists of three isoforms, α, β and γ [4–6]. In addition to the synthesis of PtdIns4,5P_2 via the 4-phosphate 5-kinase pathway, a 5-phosphate 4-kinase reaction exists and is catalysed by the type II PIPkin family consisting of several isoforms [5–7]. This route of PtdIns4,5P_2 synthesis is not well understood and it probably contributes to only a very small proportion of the total PtdIns4,5P_2 formed. Finally, the PIPkin family is completed with the type III family which are 3-phosphate 5-kinases which are responsible for phosphatidylinositol 3,5-bisphosphate (PtdIns3,5P_2) formation [8,9]. In spite of the definitions ascribed to the type I and type II PIPkin isoforms in terms of their substrate specificities and phosphorylated reaction products, in vitro assays using recombinant proteins have demonstrated promiscuous enzymatic activity [10,11]. Because of this promiscuity, caution should be exercised when trying to extrapolate these results to in vivo phosphoinositide metabolism.

During the cloning and biochemical characterisation of the type I PIPkin family members it was observed that their in vitro lipid kinase activity was enhanced in the presence of phosphatidic acid (PtdOH) [4,12–14]. Interest in the lipid second messenger PtdOH has been intense as it arises principally from two major signal transduction pathways, via phospholipase D (PLD) [15,16] and diacylglycerol kinase (DGK) [17–19]. Despite the fact that a large number of studies have implicated PtdOH as a regulator of numerous enzymes and processes [20], very few reports have come up with definitive answers. We have re-investigated these observations and in doing so moved to a physiological in vivo model system. Our results show that PLD- and DGK-derived increased intracellular PtdOH may stimulate the in vivo lipid kinase activity of type Iα PIPkin and therefore the latter represents a novel PtdOH effector.

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2. Materials and methods

2.1. Materials

[γ^{32}P]ATP (specific activity 3000 Ci/mmol) and carrier-free [^{32}P]orthophosphate were purchased from Amersham. Foetal calf serum (FCS), cell culture media and supplements were bought from Gibco. Silica gel thin layer chromatography (TLC) plates (60 Å, LK6D) were from Whatman. Lyso-PtdOH and phosphatidylinositol 4-monophosphate (PtdIns4P) was purchased from Avanti Polar Lipids. Phosphatidylinositol 3-monophosphate (PtdIns3P) was from Bio-Mol. Phorbol myristoyl acetate (PMA), PtdIns4,5P_2, PtdOH (mixed fatty acid molecular species, egg yolk) and dioctanoyl-PtdOH (diC8-PtdOH) were from Sigma. The anti-haemagglutinin (HA) antibody was purchased from the Berkeley Antibody Company, whereas the anti-protein disulphide isomerase (PDI) antibody was from StressGen Biotechnologies. DNA encoding murine type Iα PIPkin (a kind gift from Dr H. Ishihara, University of Tokyo and Prof. Y. Oka, Yamaguchi University, Japan), murine DGKα, the kinase-dead murine DGKα mutant, porcine DGKα (a kind gift from Drs H. Kanoh and F. Sakane, Sapporo Medical University School of Medicine, Sapporo, Japan) and human DGKε (a kind gift from Dr S.M. Prescott, Huntsman Cancer Center Institute, University of Utah, Salt Lake City, UT, USA) were subcloned into the pcDNA3 eukaryotic expression vector. All proteins, except for porcine DGKα, were labelled with a 5′-HA tag in order to assess their level of expression by Western blotting. The level of porcine DGKα expression was monitored by the use of a DGKα antibody (a kind gift from Dr H. Kanoh). Authentic radiolabelled standards for strong ion exchange high performance liquid chromatography (SAX-HPLC) were prepared as previously described [21]. Salts, organic solvents and other reagents were of analytical grade and were supplied by Merck.

2.2. Cell culture and transfection

Porcine aortic endothelial (PAE) cells were maintained in monolayer cultures in 100 mm diameter petri dishes in Ham's F12 medium supplemented with 10% v/v FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES. Cells were passaged twice weekly and plated into 100 mm diameter petri dishes for transfection (20 μg DNA/10^7 cells) by electroporation (Gene Pulser, Bio-Rad). For immunofluorescence and in vitro kinase assays, cells were used approximately 15 h after transfection. For cell stimulations, cell monolayers were washed twice with phosphate-buffered saline (PBS) 10 h after transfection. Thereafter, the cells were left in Ham's F12 medium containing 0.1% w/v essentially fatty acid-free bovine serum albumin (BSA) for a further 16 h.

2.3. Immunofluorescence confocal microscopy

After transfection the cells were grown on coverslips for 15 h. They were then washed with PBS and fixed at 4°C with 4% w/v paraformaldehyde in PBS for 20 min followed by blocking with 2% w/v BSA in PBS for 2 h. All the following incubations and washes were performed in the buffer 0.5% w/v BSA, 0.1% v/v Triton X-100 in PBS. The incubations with the antibodies were performed at 37°C in a moist chamber for 1 h. The anti-HA antibody was detected using a cy2-linked anti-mouse antibody and the anti-PDI antibody using a cy3-linked anti-rabbit antibody. The stained cells were observed in a confocal microscope (Leica).

2.4. Cell stimulation and PIPkin activity measurements

Non-quiescent transfected PAE cells were collected from the petri dishes by trypsinisation. Pelleted cells were frozen on dry ice before in vitro kinase assays. Quiescent PAE cells were stimulated with 10 μM lyso-PtdOH or 100 μM diC8-PtdOH for 10 min. In some experiments, the cells were pre-incubated (for 15 min) with 30 mM butan-1-ol before stimulation. After rapid aspiration of the incubation media, the cell monolayers were rapidly frozen by placing the dishes in a dry ice/ethanol mixture. The cells were collected by scraping in ice-cold PBS, followed by their lysis in ice-cold buffer containing 150 mM NaCl, 50 mM NaF, 1% v/v NP-40, 1 mM EDTA, 1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulphonyl fluoride for 15 min. After lysate clearing by centrifugation at 14 000 rpm for 15 min at 4°C in a bench microfuge, immunoprecipitation of HA-tagged enzymes was performed for 2 h at 4°C with 5 μg anti-HA antibody followed by the addition of γ-bind Sepharose (Pharmacia) for 1 h at 4°C. The immunoprecipitates were washed twice with lysis buffer, twice with 500 mM LiCl and twice with 50 mM Tris–HCl, 0.1 mM EDTA pH 7.4 (assay buffer). The effects of PtdOH, diC8-PtdOH and lyso-PtdOH on type Iα PIPkin activity was assessed by adding the lipids as a 10 times concentrate (800 μM, in 5 μl) prepared by sonication (5 min, room temperature) in assay buffer. Control preincubations employed sonicated assay buffer. PtdIns4P (the substrate) was prepared as a 10 times concentrate (in 5 μl) by its sonication (5 min, at room temperature) into assay buffer. Final assay conditions contained PtdIns4P at 200 μM, ATP at 20 μM, $MgCl_2$ at 10 mM and 10 μCi [γ^{32}P]ATP in a total volume of 50 μl. Reactions were performed at room temperature for 15 min before lipid extraction using $CHCl_3$/MeOH/HCl. The washed $CHCl_3$ phase was either directly applied to a 1% potassium oxalate-treated TLC plate followed by its development in propanol-1-ol/2 M acetic acid (2/1, v/v) or processed for SAX-HPLC (to determine the isomer of PtdInsP_2 formed) as previously described [21]. Radioactive spots on the TLC plates were identified by autoradiography followed by phosphorimaging analysis (Bio-Rad). The migration of standard authentic non-radioactive standards applied to all TLC plates was revealed by iodine staining.

2.5. PLD assay

Quiescent PAE cells in six-well plates were briefly metabolically labelled with orthophosphate (100 μCi/ml, 2 h) in phosphate-free culture medium. After a preincubation with 30 mM 1-butanol, the cells were stimulated with 100 nM PMA, 10 μM lyso-PtdOH or 100 μM diC8-PtdOH for 10 min. The overlying medium was rapidly removed before the incubations were quenched with ice-cold methanol. Total phospholipids were extracted. Phosphatidylbutanol (PtdBut) was separated from all other phospholipids by TLC using the solvent system consisting of chloroform/methanol/acetic acid (65/15/2, v/v). Radioactive spots on the TLC were identified by autoradiography followed by phosphorimaging analysis.

2.6. Western blotting

Ten per cent of the HA immunoprecipitate was mixed with sample buffer, boiled for 5 min and then subjected to SDS–PAGE. After transfer to a nitrocellulose membrane (Bio-Rad) and blocking, the membrane was probed with the primarily antibody (anti-HA or anti-DGKα). After washing, the secondary antibody (anti-horseradish peroxidase) was added. Chemical visualisation of the immobilised proteins was achieved by using an ECL kit (Amersham) according to the recommended procedures.

3. Results and discussion

In order to study the biochemical regulation of type Iα PIPkin activity, our first goal was to transfect the HA-tagged form of the enzyme in the adherent PAE cell line. A high level of expression of HA-tagged type Iα PIPkin was found after approximately 15 h after transfection. Immunofluorescence confocal microscopy clearly demonstrated that the HA-tagged type Iα PIPkin was exclusively localised at the plasma membrane (Fig. 1A). No localisation of the HA-tagged type Iα PIPkin was found within the endoplasmic reticulum as a specific antibody for the endoplasmic reticulum failed to colocalise with the anti-HA antibody (Fig. 1A). These results are in contrast to those seen in rat kidney fibroblasts where type Iα PIPkin was seen as 'nuclear speckles' free from membrane structures [22]. Confirmation of the strong expression of HA-tagged type Iα PIPkin in PAE cells was also observed by Western blotting using anti-HA antibodies. Only those cells transfected with HA-type Iα PIPkin expressed a protein with a molecular weight of approximately 68 kDa corresponding to that of type Iα PIPkin [5] (Fig. 1B). When HA immunoprecipitates from empty vector- and HA-tagged type Iα PIPkin vector-transfected PAE cells were mixed with PtdIns4P and [γ^{32}P]ATP, only those cells which had been transfected with HA-tagged type Iα PIPkin were able to phos-

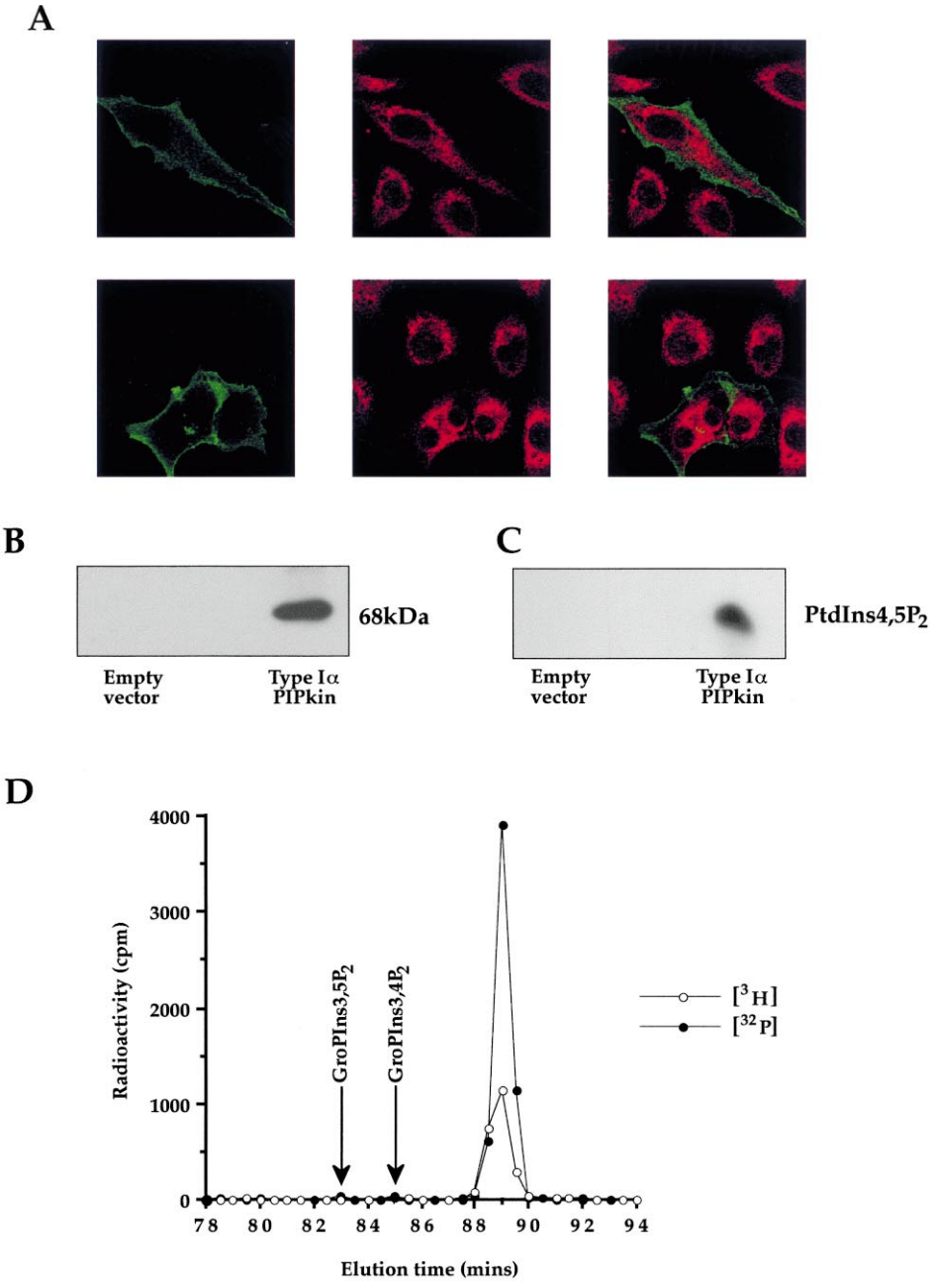

Fig. 1. Subcellular localisation, expression and in vitro lipid kinase activity of HA-tagged type Iα PIPkin. A: Immunofluorescence confocal microscopy of HA-tagged type Iα PIPkin-transfected PAE cells. HA-tagged type Iα PIPkin staining is shown in green whereas the anti-PDI staining is in red. Areas of colocalisation would merge to a yellow colour. Two fields are shown. B: Empty vector- or HA-tagged type Iα PIPkin-transfected PAE cells were lysed and HA immunoprecipitates were prepared. 10% of the HA immunoprecipitates were mixed with sample buffer before boiling for 5 min. Proteins were separated by SDS–PAGE. Western blotting was performed using the primary antibody anti-HA. C: The remainder of the HA immunoprecipitates were mixed with PtdIns4P and [γ^{32}P]ATP for the in vitro assay. After lipid extraction the radiolabelled products were separated by TLC. The panel shows the part of the TLC plate corresponding to where the authentic PtdIns4,5P_2 standard migrated. D: SAX-HPLC analysis of the radiolabelled product from the in vitro lipid kinase activity of HA-tagged type Iα PIPkin. ^{3}H radioactivity from the deacylated PtdIns4,5P_2 authentic standard is indicated by the open circles whereas the black circles represent the ^{32}P radioactivity from the in vitro kinase radiolabelled deacylated product. The arrows indicate where the deacylated PtdIns3,5P_2 authentic standard (GroPIns3,5P_2) and deacylated PtdIns3,4P_2 authentic standard (GroPIns3,4P_2) migrated in the SAX-HPLC profile. The results shown in each panel are from a single experiment representative of at least three further independent experiments.

phorylate PtdIns4P in an in vitro assay (Fig. 1C). In order to exclude any possibility that the radiolabelled product of the reaction was not PtdIns4,5P_2, the radiolabelled product was deacylated and subjected to SAX-HPLC. Fig. 1D shows that the radiolabelled reaction product coincided with an authentic deglycerated [^{3}H]PtdIns4,5P_2 standard and was well separated from the deglycerated radiolabelled standards PtdIns3,5P_2 and phosphatidylinositol 3,4-bisphosphate (PtdIns3,4P_2). When PtdIns3P instead of PtdIns4P was supplied as a substrate in other in vitro lipid kinase assays using HA-tagged type Iα PIPkin immunoprecipitates, PtdIns3,5P_2 was confirmed to be the radiolabelled product (by SAX-HPLC)

(data not shown). This observation was identical to that seen by two other independent laboratories [10,11]. These preliminary results confirmed that we had successfully transfected type Iα PIPkin into PAE cells and that the expressed protein had functional lipid kinase activity.

Our next objective was to determine if the lipid kinase activity of the expressed HA-tagged type Iα PIPkin could be modified by lipids in an in vitro assay. Fig. 2 indicates that a commercially available PtdOH preparation (mixed fatty acid molecular species from egg yolk) was able to stimulate the lipid kinase activity of type Iα PIPkin by approximately 2.1-fold. A lower fold activation (approximately 1.8 times) was observed with diC8-PtdOH (Fig. 2). Our PtdOH-dependent fold stimulation of lipid kinase activity was considerably less than that reported previously [4,12–14]. These authors used 0.1% v/v Triton X-100 in the assay. We avoided the used of detergent as this was found to greatly inhibit the basal lipid kinase activity (data not shown). In contrast to PtdOH, lyso-PtdOH failed to stimulate lipid kinase activity above that found in appropriate control incubations (Fig. 2). The results of these in vitro lipid kinase assays would suggest that activation of type Iα PIPkin requires a lipid species consisting of at least a structure containing PtdOH and therefore forms a novel mechanism for the positive modulation of the lipid kinase activity.

In PAE cells, a comprehensive study of the molecular species of DAG and the fatty acid composition of PtdOH from resting and agonist-stimulated cells has been published [23]. In this study, lyso-PtdOH was shown to deliver its mitogenic signal by increasing the intracellular PtdOH level solely through the activation of PLD. Therefore, in order to build on the observations herein and those elsewhere [4,12–14] of the stimulatory effect of PtdOH on type Iα PIPkin in vitro lipid kinase activity, we decided to determine if the lipid kinase activity of type Iα PIPkin was able to be modulated in vivo by increasing the intracellular level of PtdOH. When the HA-tagged type Iα PIPkin-transfected PAE cells were stimulated with lyso-PtdOH, approximately a 2.2-fold increase in in vitro lipid kinase activity was seen (Fig. 3A). This stimulation

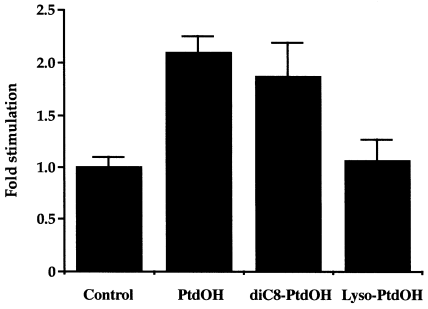

Fig. 2. PtdOH but not lyso-PtdOH is able to stimulate the lipid kinase activity of type Iα PIPkin in an in vitro assay. PAE cells were transfected with a vector containing HA-tagged type Iα PIPkin. After lysis, HA immunoprecipitates were prepared. These were then preincubated with either PtdOH (of mixed fatty acid molecular species originating from egg yolk), diC8-PtdOH or lyso-PtdOH before being mixed with PtdIns4P and [γ^{32}P]ATP for the in vitro assay. After lipid extraction, the radiolabelled products were separated by TLC. The radioactivity found within the area where the authentic unlabelled PtdIns4,5P_2 standard migrated was quantified by phosphorimaging. The figure shows the results (mean ± S.D. of duplicate determinations) of a single experiment which is representative of a further independent experiment with identical results.

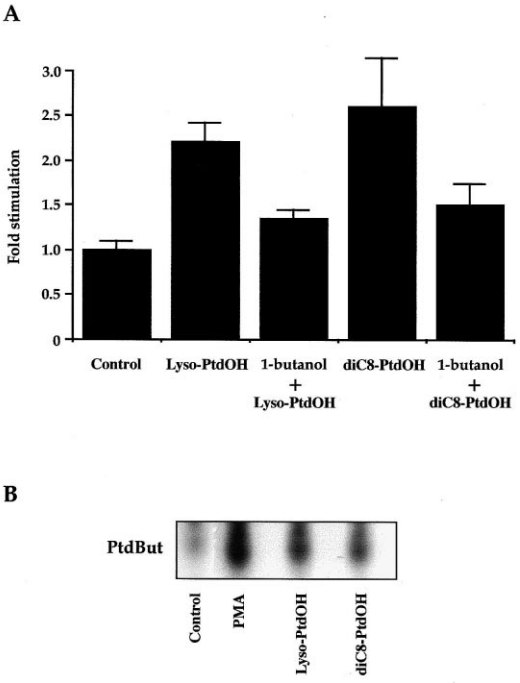

Fig. 3. Type Iα PIPkin lipid kinase activity is increased in vivo by stimulation of PAE cells with lyso-PtdOH and diC8-PtdOH via PLD. A: PAE cells were transfected with a vector containing HA-tagged type Iα PIPkin and then left to enter a state of quiescence. Half the dishes of cells were then treated with 30 mM 1-butanol. Afterwards the cells were stimulated with lyso-PtdOH and diC8-PtdOH. After freezing the cell monolayers, the cells were collected by scraping. Thereafter, HA immunoprecipitates were prepared and mixed with PtdIns4P and [γ^{32}P]ATP for the in vitro assay. After lipid extraction, the radiolabelled products were separated by TLC. The radioactivity found within the area where the authentic unlabelled PtdIns4,5P_2 standard migrated was quantified by phosphorimaging. The panel shows the results (mean ± S.D. of duplicate determinations) of a single experiment which is representative of a further independent experiment with identical results. B: Non-transfected PAE cells were left to enter a state of quiescence during which metabolic radiolabelling with orthophosphate was performed during the last 2 h. The cells were then treated with 30 mM 1-butanol before stimulations with PMA, lyso-PtdOH and diC8-PtdOH. After quenching the incubations, total phospholipids were extracted followed by their separation by TLC. The panel shows the part of the TLC plate corresponding to where the authentic PtdBut standard migrated. The panel shows the results (mean ± S.D. of duplicate determinations) of a single experiment which is representative of a further independent experiment with identical results.

in lipid kinase activity was inhibited (by approximately 70%) by prior treatment of the cells with 1-butanol which is known to block the production of PtdOH via PLD by converting it into phosphatidylbutanol through the transphosphatidylation reaction [15,16]. This result shows for the first time that agonist-stimulated PtdOH production is able to stimulate the lipid kinase activity of type Iα PIPkin in vivo. When HA-tagged type Iα PIPkin-transfected PAE cells were stimulated with diC8-PtdOH, a slightly higher increase in lipid kinase activity (approximately 2.6-fold) was observed when measured in the in vitro assay (Fig. 3A). It has been reported that PtdOH can stimulate PLD activity [24]. Therefore, an increase in endogenous PtdOH may mediate cell stimulation by exogenously added diC8-PtdOH. Support for this claim is shown in the same panel as significant inhibition (approximately 70%) of stimulated lipid kinase activity was seen when the

cells were preincubated with 1-butanol prior to their stimulation with diC8-PtdOH. Confirmation of the activation of PLD by diC8-PtdOH is indicated in Fig. 3B. The stimulated fold increases in PLD activity, as measured by the transphosphatidylation reaction, for PMA (positive control), lyso-PtdOH and diC8-PtdOH were approximately four, three and two times above control, respectively.

In PAE cells the fatty acid composition of resting and agonist-stimulated PtdOH consists of essentially saturated and monounsaturated fatty acids [23]. In our in vitro lipid kinase assays we used a commercial preparation of PtdOH, of mixed fatty acid composition. The fatty acid analysis of the PtdOH preparation by gas chromatography/mass spectrometry revealed that it consisted mainly of saturated and monounsaturated fatty acids (palmitic acid, stearic acid and oleic acid) with some diunsaturated fatty acid (linoleic acid) (Jones and Mérida, unpublished observations). As there were similarities in the compositions of both the lyso-PtdOH-stimulated PtdOH accumulation in PAE cells [23] and the PtdOH used in the in vitro assay, together the results point to the fact that a saturated/monounsaturated PtdOH may be the preferred activator of type Iα PIPkin in vivo.

In recent years there has been increasing interest in the DGK family of enzymes and their role in signal transduction [17–19]. As DGK is a key regulator of the levels of both DAG and PtdOH, we decided to ask the question if PtdOH-derived from DGK was also able to stimulate type Iα PIPkin lipid kinase activity in vivo. Because of the scarcity of extracellular stimuli that stimulate DGK activity, we opted to raise the level of PAE cell PtdOH by transfection with the α isoform of porcine DGK or with the recently cloned α isoform of murine DGK (GenBank accession number AF085219, Sanjuan, Jones and Mérida, submitted for publication) in combination with HA-tagged type Iα PIPkin. When the HA-tagged type Iα PIPkin was co-transfected with either the porcine α or murine α DGK isoform, a small increase (approximately 30% above empty vector control co-transfection) in the type Iα PIPkin lipid kinase activity was found in anti-HA immunoprecipitates in an in vitro assay (Fig. 4). No such increases in type Iα PIPkin lipid kinase activity in the in vitro assay were seen in a co-transfection of HA-tagged type Iα PIPkin with a kinase-dead mutant of the murine α DGK isoform (Sanjuan, Jones and Mérida, submitted for publication) (Fig. 4). This result with the kinase-dead mutant of murine DGKα confirms that the production of PtdOH is essential for the stimulation of type Iα PIPkin lipid kinase activity in vivo rather than a PtdOH-independent stimulation of the lipid kinase activity which could be just through association of proteins leading to changes in their conformation or localisation. Interestingly, a co-transfection of HA-tagged type Iα PIPkin with the human DGKε isoform did not lead to an increase in type Iα PIPkin lipid kinase activity in HA immunoprecipitates in the in vitro assay (Fig. 4). In PAE cells there is a large amount of polyunsaturated DAG (over 25% of total DAG) [23]. In vitro studies have shown that the DGKε isoform is the only DGK isoform which shows specificity for a DAG containing a polyunsaturated fatty acid [25–27]. This specificity has been the subject of a very recent in vivo study confirming the selective removal of solely polyunsaturated DAG species by DGKε in stably DGKε-transfected PAE cells [28].

Recent progress in the molecular species analysis of the lipid second messengers DAG and PtdOH has demonstrated

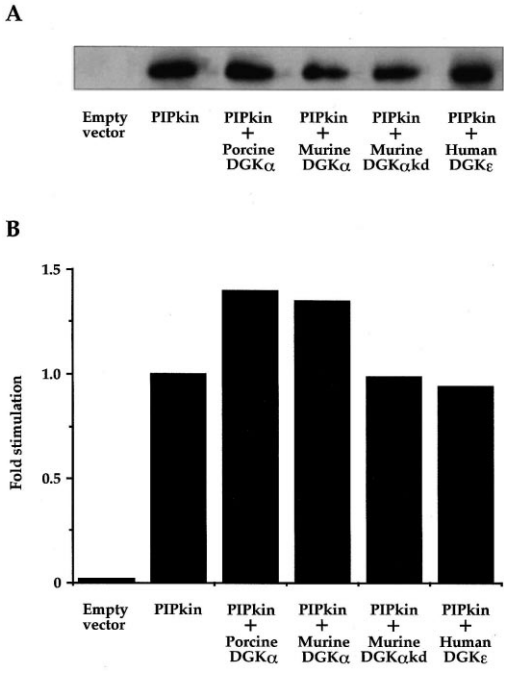

Fig. 4. Type Iα PIPkin lipid kinase activity is increased in vivo by DGKα isoform-derived PtdOH. A: HA-tagged type Iα PIPkin was co-transfected with either the porcine DGKα isoform, the murine DGKα isoform, a kinase-dead (kd) murine DGKα isoform or the human DGKε isoform into PAE cells. After 15 h the cells were lysed and HA immunoprecipitates were prepared. 10% of the immunoprecipitates were mixed with sample buffer before boiling for 5 min. Proteins were separated by SDS–PAGE. Western blotting was performed using the primary antibody anti-HA. The panel shows the expression of the HA-tagged type Iα PIPkin in co-transfected cells from a single experiment which is representative of a further independent experiment with identical results. B: The remainder of the HA immunoprecipitates were mixed with PtdIns4P and [γ^{32}P]ATP for the in vitro assay. After lipid extraction the radiolabelled products were separated by TLC. The radioactivity found within the area where the authentic unlabelled PtdIns4,5P_2 standard migrated was quantified by phosphorimaging. The panel shows the results of a single experiment which is representative of a further independent experiment with identical results.

that polyunsaturated DAG is generally the more biologically active form of this second messenger (compared to saturated/monounsaturated DAG) whereas in the case of PtdOH the reverse is true [23,29–32]. This therefore emphasises the fact that PtdOH produced by the DGKα isoform has a strong signalling potential, far greater than that produced by DGKε. These results underline the importance of the generation of biologically active lipid second messengers for the propagation of signal transduction pathways and for cross-talk between otherwise separate pathways. Currently we are unaware how PtdOH stimulates type Iα PIPkin lipid kinase activity in PAE cells. A change in the localisation of type Iα PIPkin was not seen when the cells were stimulated with lyso-PtdOH (data not shown). One possibility is that type Iα PIPkin may associate with other proteins/enzymes such as small GTPases [33–37], protein kinase Cμ [38], PLD [39] and type II phosphatidylinositol 4-kinase [38,39] thereby rapidly regulating its activity. Another mechanism for type Iα PIPkin regulation could be that of covalent modification such as phosphorylation or dephosphorylation events. In summary, our results show that PtdOH derived either via PLD or via DGKα may stimulate the activity of type Iα PIPkin for the

enhanced production of PtdIns4,5P_2 and that the latter enzyme is the first in vivo physiological target identified for the lipid second messenger PtdOH.

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