THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 45, pp. 31062–31073, November 6, 2009

Diacylglycerol Kinase ϵ Is Selective for Both Acyl Chains of Phosphatidic Acid or Diacylglycerol*

Received for publication, July 30, 2009, and in revised form, August 31, 2009 Published, JBC Papers in Press, September 10, 2009, DOI 10.1074/jbc.M109.050617

Michael Lung[‡1], Yulia V. Shulga[‡], Pavlina T. Ivanova[§], David S. Myers[§], Stephen B. Milne[§], H. Alex Brown[§], Matthew K. Topham[¶], and Richard M. Epand[‡2]

From the [‡]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada, the [§]Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232, and the [¶]Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112

The phosphatidylinositol (PI) cycle mediates many cellular events by controlling the metabolism of many lipid second messengers. Diacylglycerol kinase ϵ (DGKϵ) has an important role in this cycle. DGKϵ is the only DGK isoform to show inhibition by its product phosphatidic acid (PA) as well as substrate specificity for *sn*-2 arachidonoyl-diacylglycerol (DAG). Here, we show that this inhibition and substrate specificity are both determined by selectivity for a combination of the *sn*-1 and *sn*-2 acyl chains of PA or DAG, respectively, preferring the most prevalent acyl chain composition of lipids involved specifically in the PI cycle, 1-stearoyl-2-arachidonoyl. Although the difference in rate for closely related lipid species is small, there is a significant enrichment of 1-stearoyl-2-arachidonoyl PI because of the cyclical nature of PI turnover. We also show that the inhibition of DGKϵ by PA is competitive and that the deletion of the hydrophobic segment and cationic cluster of DGKϵ does not affect its selectivity for the acyl chains of PA or DAG. Thus, this active site not only recognizes the lipid headgroup but also a combination of the two acyl chains in PA or DAG. We propose a mechanism of DGKϵ regulation where its dual acyl chain selectivity is used to negatively regulate its enzymatic activity in a manner that ensures DGKϵ remains committed to the PI turnover cycle. This novel mechanism of enzyme regulation within a signaling pathway could serve as a template for the regulation of enzymes in other pathways in the cell.

Diacylglycerol kinases (DGK[3]; EC 2.7.1.107) are a diverse family of lipid kinases that catalyze the phosphorylation of diacylglycerol (DAG) to phosphatidic acid (PA) using ATP as a phosphate donor (1–8). DAG is a lipid second messenger whose importance in cell signaling is well established (9). DAG is generated through the catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) by the various isoforms of phospholipase C in response to cell stimulation by various agonists such as growth factors, cytokines, and hormones (9, 10). The diverse range of effectors of DAG allows it to modulate a large variety of cellular events, resulting in its broad effects on the cell. Of these effectors, DAG is most known as an allosteric activator of protein kinase C (11–14). DAG also activates other effectors such as Ras-guanyl nucleotide-releasing protein (11) and the transient receptor potential channel 2 (15). Furthermore, DAG is involved in the recruitment of chimerins (16), Munc13, and protein kinase D to the membrane (11). Because DAG is such an important signaling molecule, there must be tight regulation of its concentration. The DGK-catalyzed phosphorylation of DAG to PA serves as one pathway for the attenuation of the pleiotropic effects of DAG.

Although the reaction catalyzed by DGK attenuates the effects of DAG, the product of the reaction, PA, is also a lipid second messenger that has its own range of effects on the cell through its own set of diverse effectors. For instance, PA regulates the activity of phosphatidylinositol-4-phosphate 5-kinase (17), phospholipase C-γ1 (18), protein kinase C-ζ (19), protein phosphatase 1 (20), and the mammalian target of rapamycin (21). PA also mediates the membrane translocation of enzymes such as sphingosine kinase (22) and Raf-1 kinase (23). Furthermore, PA mediates secretory vesicle trafficking from the Golgi (24) and stimulates DNA synthesis in fibroblast cells (25). Therefore, because DGK modulates the levels of two important lipid second messengers (substrate DAG and product PA), DGK is a critical link in cell signaling and accordingly is tightly regulated.

It has been suggested that DGK controls functions unique to multicellular organisms, as the one DGK found in bacteria and the one DGK recently found in yeast show very little sequence homology or structure similarity to the higher eukaryotic forms (26, 27) such as those found in *Drosophila melanogaster* (28–30), *Dictyostelium discoideum* (31), *Caenorhabditis elegans*

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-CA95463 (to M. K. T.) and by National Institutes of Health Large Scale Collaborative Initiative LIPID MAPS Grant U54 GM069338 (to H. A. B.). This work was also supported by Natural Sciences and Engineering Research Council of Canada Grant 9848 (to R. M. E.).

[1] Recipient of a Canada Graduate Scholarship from the Natural Sciences and Engineering Research Council of Canada.

[2] To whom correspondence should be addressed: Dept. of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main St. West, Hamilton, Ontario L8N 3Z5, Canada. Tel.: 905-525-9140; Fax: 905-521-1397; E-mail: epand@mcmaster.ca.

[3] The abbreviations used are: DGK, diacylglycerol kinase; DAG, diacylglycerol; DGKϵ, diacylglycerol kinase ϵ; MEF, mouse embryonic fibroblast; OG, octyl β-D-glucopyranoside; PA, phosphatidic acid; PC, phosphatidylcholine; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; DOG, 1,2-dioleoyl-*sn*-glycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; PAG, 1-palmitoyl-2-arachidonoyl-*sn*-glycerol; AAG, 1-arachidoyl-2-arachidonoyl-*sn*-glycerol; WT, wild type; SAPA, 1-stearoyl-2-arachidonoyl phosphatidic acid; SOPA, 1-stearoyl-2-oleoyl phosphatidic acid; PAPA, 1-palmitoyl-2-arachidonoyl phosphatidic acid; SLPA, 1-stearoyl-2-linoleoyl phosphatidic acid; SOPA, 1-stearoyl-2-oleoyl phosphatidic acid; KO, knock-out.

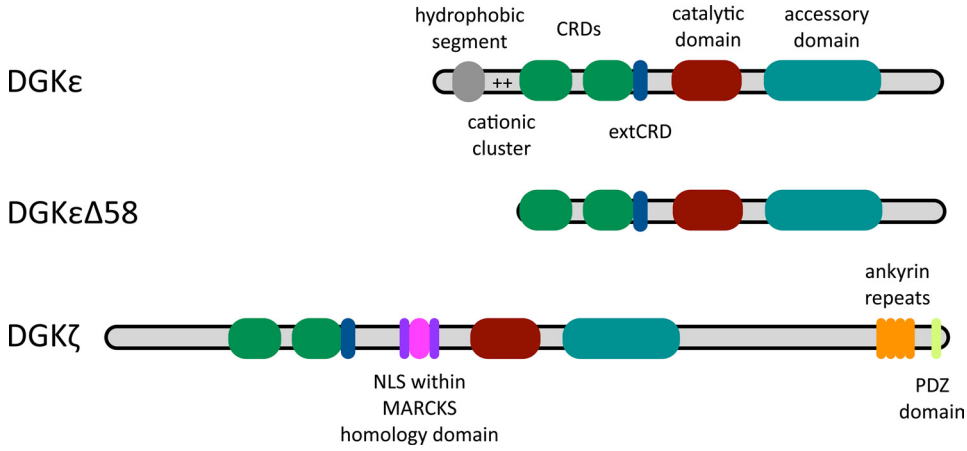


FIGURE 1. **Overview of DGKε, DGKεΔ58, and DGKζ structure.** The various domains/motifs in each of these proteins are presented here. *CRD*, cysteine-rich domain; *extCRD*, extension of the cysteine-rich domains; *MARCKS*, myristoylated alanine-rich protein kinase C substrate; *NLS*, nuclear localization sequence; *PDZ*, PSD95/DlgA/zo-1. The structures presented here are not drawn to exact scale.

(32), *Arabidopsis thaliana* (33–35), and mammals (2, 4–8). All mammalian DGKs have a conserved catalytic domain and at least two cysteine-rich domains similar to the C1 domains in protein kinase C (but lacking critical consensus residues found in protein kinase C C1) that has been predicted to bind to the substrate DAG. Ten mammalian DGK isoforms have currently been experimentally identified (α, β, γ, δ, ε, ζ, η, θ, ι, and κ) (4–8) and are classified into five groups based on a combination of various structural domains or motifs that contribute to their differing regulation, cellular localization, and subsequent cellular function (2, 4–8).

The ε isoform of mammalian DGKs (DGKε) is the smallest and simplest (in terms of primary structure) mammalian DGK known to date, with a molecular mass of 64 kDa and a lack of extra regulatory domains from which function can be inferred (36). However, it possesses many unique features that make it one of the most intriguing DGK isoforms. First, it is the only constitutively active/membrane-bound DGK isoform with a hydrophobic segment (residues 20–40 in human DGKε) (Fig. 1) that has been proposed to form a bent helix that exits the membrane through the same side it entered (8, 37, 38). It is also the only DGK isoform that shows substrate specificity *in vitro* for DAG with an arachidonoyl acyl chain at the *sn*-2 position (*sn*-2 arachidonoyl-DAG) (8, 36, 37, 39–41). These observations taken together with studies showing that phosphatidylinositol (PI) turnover cycle lipids are enriched with arachidonate suggests that DGKε is fully committed to the PI turnover cycle in the enrichment of its lipids with arachidonate through multiple iterations of this cycle. It is known that this arachidonoyl enrichment is not a result of acyl chain remodeling (42). The PI cycle mediates a large variety of cellular events by controlling the metabolism of several important lipid second messengers, including several phosphoinositides (43), DAG and PA, each possessing their own diverse range of signaling roles in the cell. This postulation of DGKε function has been supported by recent *in vivo* data in which we show that DGKε KO mouse embryonic fibroblast (MEF) cells have a significant reduction in PI with arachidonoyl side chains compared with WT cells (44).

It is instructive to compare these lipodomics results (obtained from DGKε KO MEF cells) (44) with an earlier study in which DGKε was overexpressed in porcine aortic endothelial cells (45). Overexpression of DGKε results in the selective removal of DAGs with certain acyl chains, including those with an arachidonoyl group (45). In contrast, there is no concentration or acyl chain difference among the different species of DAG when comparing WT with DGKε KO MEFs (44). Thus, although one would anticipate an increase in arachidonoyl-containing DAG in the DGKε KO MEF cells, because there are many pathways for the removal of DAG (including other isoforms of DGK), this difference is not observed. In contrast, with overexpression, those forms of DAG that are affected are virtually completely eliminated, including both 1-palmitoyl-2-arachidonoyl-*sn*-glycerol (PAG) and 1-palmitoyl-2-arachidonoyl-*sn*-glycerol (SAG). However, as we demonstrate below, differences in the level of PI made from SAG *versus* PAG are observed between the WT and DGKε KO MEFs. Thus, overexpression of DGKε results in less selective changes in lipid species compared with knocking out this enzyme.

Another aspect of DGKε that differs from other DGK isoforms is the modulation of its enzymatic activity by anionic phospholipids. Although many DGK isoforms other than DGKε show increased enzymatic activity in the presence of phosphatidylserine (PS) and other anionic phospholipids (40, 46–48), PS has been shown to strongly inhibit DGKε enzymatic activity *in vitro* (40). Interestingly, PtdIns(4,5)P_2 and PA, two molecules involved in the PI turnover cycle, have also been shown to inhibit DGKε enzymatic activity *in vitro* (40). It had been previously hypothesized that the cluster of cationic residues (residues 43, 45, 46, 50, 51, 55, 56, and 58 in human DGKε) adjacent to the hydrophobic segment of DGKε (Fig. 1) was responsible for its inhibition by PA (40). In addition, this hydrophobic segment could also contribute to the acyl chain selectivity of DGKε by accommodating the acyl chains of PA. Also, it was previously shown that in contrast to the situation for DGKε substrate specificity, an arachidonoyl-specific DGK from bovine testes did not exhibit acyl chain selectivity in its inhibition by PA (49). However, this arachidonoyl-specific DGK had an apparent molecular mass of 58 kDa that differed from the molecular mass of DGKε (64 kDa). In addition, this arachidonoyl-specific DGK from bovine testes exhibits noncompetitive inhibition of PA with respect to DAG (49), although we find the inhibition to be competitive (see below). Thus, the identity of this arachidonoyl-specific DGK from bovine testes is uncertain, and we have therefore examined the role of acyl chain specificity in the PA inhibition of DGKε.

Here, we show that the PA inhibition and substrate specificity of DGKε are both based upon a selectivity for a combination

TABLE 1
Lipids used in this study

Abbreviation	Full name	Alternative notation (*sn*-1/*sn*-2)
DAG		
AAG	1-Arachidoyl-2-arachidonoyl-*sn*-glycerol	20:0/20:4 DAG
DOG	1,2-Dioleoyl-*sn*-glycerol	18:1/18:1 DAG
PAG	1-Palmitoyl-2-arachidonoyl-*sn*-glycerol	16:0/20:4 DAG
SAG	1-Stearoyl-2-arachidonoyl-*sn*-glycerol	18:0/20:4 DAG
SLG	1-Stearoyl-2-linoleoyl-*sn*-glycerol	18:0/18:2 DAG
PA		
AAPA	1-Arachidoyl-2-arachidonoyl phosphatidic acid	20:0/20:4 PA
DAPA	1,2-Diarachidonoyl phosphatidic acid	20:4/20:4 PA
PAPA	1-Palmitoyl-2-arachidonoyl phosphatidic acid	16:0/20:4 PA
POPA	1-Palmitoyl-2-oleoyl phosphatidic acid	16:0/18:1 PA
SAPA	1-Stearoyl-2-arachidonoyl phosphatidic acid	18:0/20:4 PA
SLPA	1-Stearoyl-2-linoleoyl phosphatidic acid	18:0/18:2 PA
SOPA	1-Stearoyl-2-oleoyl phosphatidic acid	18:0/18:1 PA
PC		
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	18:1/18:1 PC
PS		
DOPS	1,2-Dioleoyl-*sn*-glycero-3-[phospho-L-serine]	18:1/18:1 PS

of their *sn*-1 and *sn*-2 acyl chains, preferring the most abundant acyl chain composition of PI turnover cycle lipids, contrasting to other DGKs such as DGKζ. In further support of the similarity between PA and DAG acyl chain selectivity, we also show that the removal of the hydrophobic segment and cationic cluster within DGKε does not affect PA or DAG acyl chain selectivity and that PA inhibition is competitive, suggesting that PA competes with DAG for the active site of DGKε.

EXPERIMENTAL PROCEDURES

Reagents and General Notes—Chemicals and reagents were purchased from Sigma or BioShop Canada unless otherwise stated. All lipid stocks were purchased from Avanti Polar Lipids containing 0.1% (w/v) butylated hydroxytoluene and were stored either in 2:1 $CHCl_3/CH_3OH$ or in $CHCl_3$. The butylated hydroxytoluene was eliminated during solvent evaporation and was not present in the enzymatic assay. Furthermore, all lipid films were immediately covered with argon gas after solvent evaporation to protect them from contact with air. All lipids (their abbreviations, full names, and alternative notations) used in this study are listed in Table 1. All antibodies were purchased from Santa Cruz Biotechnology unless otherwise stated. Reagents used in PCR and DNA digestion reactions were purchased from Fermentas Life Sciences. When the acronym DAG is used in this study, it is solely referring to the 1,2-diacylglycerol form of diacylglycerol. When human DGKε amino acid residue numbers are listed in this study, the first methionine amino acid residue is not counted.

Quantification of Phosphatidic Acid—The concentration of all PA stocks used in this study was determined experimentally based on an assay for inorganic phosphate as described previously (50). Briefly, 30 μl of 10% (w/v) $Mg(NO_3)_2$ in 95% ethanol was added to an aliquot of PA (up to 80 nmol) in an acid-washed Pyrex tube, which was subsequently flamed until the organic phosphate was completely ashed. 350 μl of 0.5 N HCl was then added, and the mixture was subsequently incubated at 100 °C for at least 15 min. Afterward, 750 μl of a 1:6 mixture of 10% (w/v) L-ascorbic acid, 0.42% (w/v) ammonium molybdate tetrahydrate in 1 N H_2SO_4 was added. The mixture was then incubated at 60 °C for 10 min and allowed to cool to room temperature where its absorbance was read at 820 nm.

Quantification of Diacylglycerol—The concentration of all DAG stocks used in this study was quantified experimentally. 100 μl of 50 mM tetraethylammonium hydroxide in 100% ethanol was added to a borosilicate test tube containing ~50 nmol of DAG. The tube was subsequently incubated at 60 °C for 30 min. After the incubation, 100 μl of 0.1 N HCl and 2 ml of hexane was added to each of the tubes, where the top phase containing the free fatty acid was then eliminated. Aliquots of the bottom phase were taken where the presence of free glycerol was quantified using the EnzyChrom glycerol assay kit (BioAssay Systems) according to the manufacturer's instructions.

Construction of p3XFLAG-DGKεΔ58 Mammalian Expression Vector—A PCR fragment lacking the N-terminal 174 bases of human *DGKε* cDNA (for expression of DGKε lacking the N-terminal 58 amino acids) was amplified from an N-terminal hemagglutinin-tagged DGKε vector using *Pfu* DNA polymerase and "polished" with *Taq* DNA polymerase. The following primers were used: forward, 5′-AGATCTGCACGGGTGGCGCGACACG-3′; reverse, 5′-TCTAGACTATTCAGTCGCCTTTATATCTTC-3′. The PCR fragment was gel-purified and cloned into a pCRII-TOPO-TA cloning vector (Invitrogen). The DNA fragment of interest was then excised from the TOPO-TA vector by digestion with the restriction enzymes BglII and XbaI, gel-purified, and subcloned into the p3XFLAG-CMV-7.1 vector (Sigma), forming the mammalian expression vector p3XFLAG-DGKεΔ58. The identity of the DNA was confirmed by sequencing analysis performed at the Huntsman Cancer Institute Sequencing Facility (Salt Lake City, UT).

Cell Culturing and Transfection of DGKε into COS-7 Cells—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37 °C and 5% atmospheric CO_2. Cells were transiently transfected with vectors for the expression of an N-terminal 3XFLAG epitope tag fusion to full length or an N-terminal 58- amino acid truncation of human DGKε (3XFLAG-DGKεFL or 3XFLAG-DGKεΔ58, respectively) (Sigma) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24–48 h after transfection, cells were scraped into ice-cold phosphate-buffered saline (1×; pH 7.2) (Invitrogen) containing a 1:1000 dilution of 1× protease inhibitor mixture (Sigma). The cells were pelleted at 8000 × *g* at 4 °C and subsequently kept at −80 °C until further use.

Enzyme Preparation for DGK Enzymatic Activity Assay—A pellet of COS-7 cells overexpressing human 3XFLAG-DGKεFL or 3XFLAG-DGKεΔ58 or a pellet of baculovirus-infected Sf21 cells overexpressing a C-terminal hexahistidine or FLAG epitope tag fusion to human DGKε (DGKε-His_6) or DGKζ (DGKζ-FLAG) proteins, respectively, was resuspended in ice-cold lysis buffer (1% (v/v) (octylphenoxy)polyethoxyethanol (Nonidet P-40), 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM activated sodium orthovanadate, and 1:1000 dilution of 1× protease inhibitor mixture) and allowed to lyse on ice for 10 min. The lysate from COS-7 or insect cells was centrifuged at 100,000 × *g* for 30 min at 4 °C. The supernatant con-

taining extracted DGK was then used for enzymatic activity assays. In this study, the term "enriched lysates" refers to this supernatant containing detergent-extracted DGK. Attempts to obtain a highly purified preparation of DGKε were unsuccessful. To our knowledge, there is no report of DGKε ever having been purified. There is an arachidonoyl-specific DGK isolated from bovine testes that has been purified to apparent homogeneity (39). However, there is evidence that this protein differs from DGKε.

Detergent-Phospholipid-Mixed Micelle-based DGK Enzymatic Activity Assay—DGK was assayed for enzymatic activity using a detergent-phospholipid-mixed micelle-based protocol adapted from previous studies (39, 40, 49). Lipid films composed of the substrate (DAG) along with any phospholipid component required in the assay (PA and/or 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (for DGKε) and/or 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (for DGKζ)) were made at a constant total lipid concentration. For example, when substrate concentrations were increased, DOPC or 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] concentrations were decreased to maintain a constant total lipid concentration. Mixed micelles were formed by hydrating these lipid films with 50 μl of 4× Assay Buffer (200 mM Tris-HCl (pH 7.5), 400 mM NaCl, 20 mM $MgCl_2$, 4 mM EGTA, 1 mM dithiothreitol) containing either 30 mM Triton X-100, 30 mM Triton X-114, or 300 mM octyl β-D-glucopyranoside (OG) and subsequently vortexing the hydrated lipid film for 2 min. Enriched lysates from Sf21 or COS-7 cells expressing DGK were added to the mixed micelles along with double distilled H_2O to a final volume of 180 μl. The reaction was initiated by adding 20 μl of 1 mM [γ-^{32}P]ATP (50 μCi/ml) (PerkinElmer Life Sciences), allowed to proceed for 10 min at 25 °C, and terminated with 2 ml of stop solution (1:1 $CHCl_3/CH_3OH$, 0.25 mg/ml dihexadecyl phosphate). The organic layer was washed three times with 2 ml of wash solution (7:1 H_2O/CH_3OH, 1% $HClO_4$, 0.1% H_3PO_4). An aliquot of the organic layer was used to quantify the incorporation of ^{32}P into PA using Cerenkov counting. All enzymatic activity data presented in this study should be considered as data obtained from initial rate experiments, because the formation of the product PA was linear over the 10-min reaction period (data not shown). The assays were performed in triplicate and will be presented in this study as the mean ± S.D. Enriched lysates from mock-transfected cells (COS-7 or Sf21) were always used as a negative control and were confirmed to have activity levels significantly below enriched lysates from cells overexpressing DGK. All results presented in this study were repeatable over multiple independent experiments, consistently showing the same order of effects among different lipids. Because DGKs are interfacial enzymes in that they perform catalysis at a lipid-aqueous interface such as the surface of the detergent-phospholipid-mixed micelle, the concentrations of the individual lipid components at the surface of this mixed micelle are important in affecting DGK enzymatic activity rather than the bulk concentrations of the lipid components (51, 52). Thus, the concentrations of the individual lipid components of the mixed micelles will be listed when appropriate in this study as their mole percentage of the detergent-phospholipid-mixed micelle.

Kinetic Analysis of DGKε Inhibition by PA—Two methods of kinetic analysis were utilized to determine the type of enzyme inhibition used by PA in its inhibition of DGKε. The first method employed was an analysis of the kinetic data using a Cornish-Bowden plot ((substrate concentration) (initial velocity)$^{-1}$ *versus* inhibitor concentration). The second method employed was a nonlinear regression analysis, where the kinetic data were fitted to nonlinear expressions for different types of enzyme inhibition using the SigmaPlot data analysis software program (version 11), Enzyme Kinetics module (Systat Software). This nonlinear regression analysis (with fitting of the kinetic data to a nonlinear expression for a competitive type of enzyme inhibition because PA inhibition of DGKε was determined to be competitive, see "Results") was also used to determine the values of the kinetic constants K_i and K_m.

Immunoblot Analysis—Protein samples for immunoblot analysis were prepared by incubation at 95 °C for 10 min. The resultant proteins were separated by 7.5% Tris-glycine SDS-PAGE and electroblotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore). The membrane was then incubated with either a 1:2500 dilution of mouse anti-FLAG M2 (Sigma) or 1:500 dilution of goat anti-actin (sc-1616) as the primary antibody and either a 1:2500 dilution of horseradish peroxidase-conjugated goat anti-mouse (sc-2005) or donkey anti-goat antibody (sc-2020) as the secondary antibody. The antibody complexes were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and X-Omat LS film (Eastman Kodak Co.) according to the manufacturer's instructions.

Glycerophospholipid Analysis—Phospholipids from DGKε KO and WT MEFs were extracted by a modified Bligh-Dyer extraction procedure using acidified methanol as described before (53). Mass spectral analysis was performed by direct infusion on a Finnigan TSQ Quantum triple-quadrupole mass spectrometer essentially as described previously (44). Identification of the individual phospholipids was accomplished by liquid chromatography-tandem mass spectrometry by utilization of Applied Biosystems/MDS SCIEX 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-μm particle size) using a gradient elution as communicated elsewhere (44, 53). The identification of the individual species was based on their chromatographic and mass spectral characteristic (44, 53). This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn*-1 *versus sn*-2).

Fatty Acid Analysis of DGKε KO Versus WT MEF Phospholipid Extracts—Glycerophospholipids containing stearoyl fatty acid chains were identified by liquid chromatography-tandem mass spectrometry. The sums of the intensities of the stearoyl-containing lipids within each lipid subclass (PA, PI, PS, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol) were compared (Student's *t* test) between DGKε KO and WT MEFs.

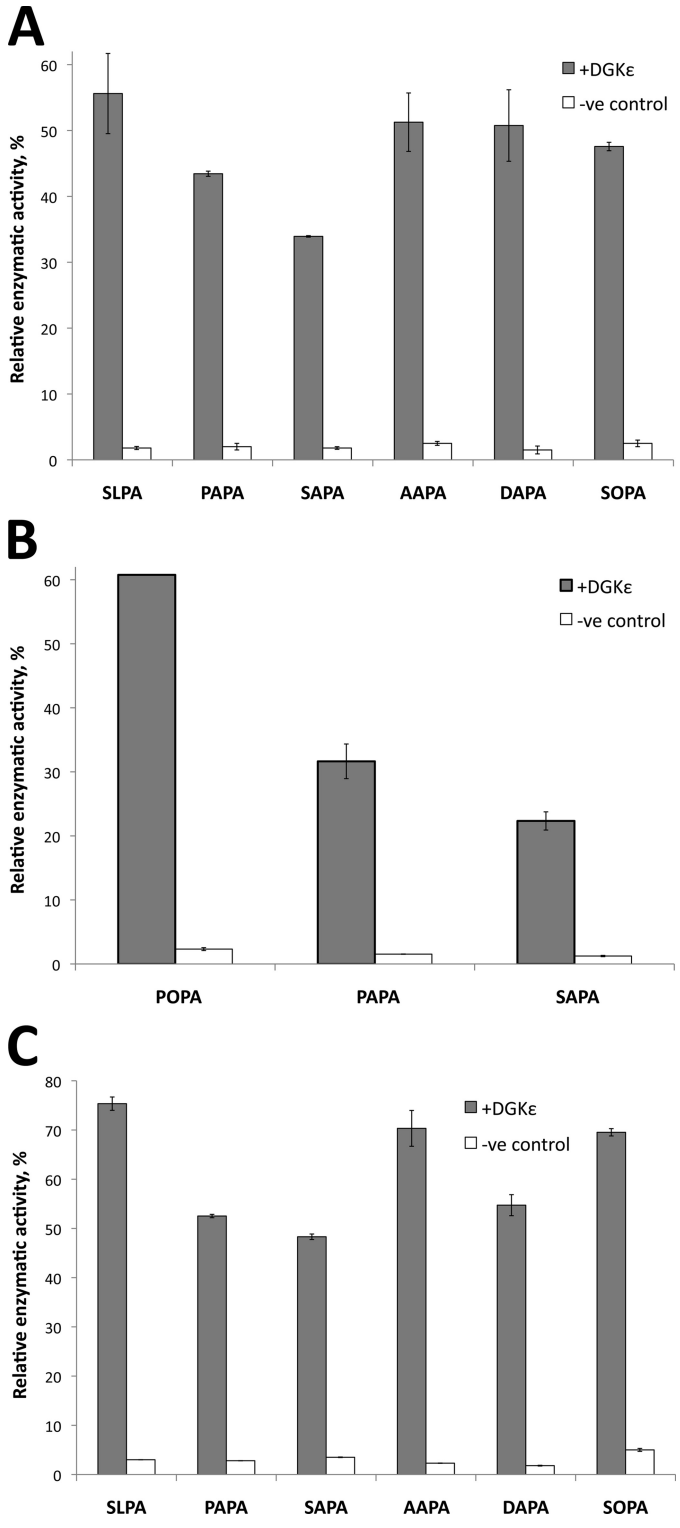


FIGURE 2. **DGKε inhibition by PA is dependent upon both its *sn*-1 and *sn*-2 acyl chains.** *A*, enriched lysates from Sf21-overexpressing DGKε-His_6 (+*DGKε*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity with 7.5 mM Triton X-100, 7.5 mM Triton X-114, 0.1 mM [γ-^{32}P]ATP, and 1.5 mol % SAG in the presence of either 1.5 mol % SLPA, PAPA, SAPA, 1-arachidoyl-2-arachidonoyl phosphatidic acid (*AAPA*), 1,2-diarachidonoyl phosphatidic acid (*DAPA*), or SOPA. The enzymatic activity presented was normalized to DGKε enzymatic activity in the absence of PA, which was 0.932 ± 0.080 nmol of PA/min and defined as 100% enzymatic activity. *B*, enriched lysates from Sf21-overexpressing DGKε-His_6 (+*DGKε*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity as in *A* except either 1.5 mol % 1-palmitoyl-2-oleoyl phosphatidic acid (*POPA*), PAPA, or SAPA was used instead of the PAs used in *A*. The enzymatic activity presented was normalized to DGKε enzymatic activity in the absence of PA, which was 0.873 ± 0.001 nmol of PA/min and defined as 100% enzymatic activity. *C*, enriched lysates from Sf21-overexpressing DGKε-His_6 (+*DGKε*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity as in *A* except 3.0 mol % 1-stearoyl-2-linoleoyl-*sn*-glycerol (*SLG*) was used instead of SAG as the DAG lipid substrate, and 0.75 mol % of the PAs was used. The enzymatic activity presented was normalized to DGKε enzymatic activity in the absence of PA, which was 0.266 ± 0.005 nmol of PA/min and defined as 100% enzymatic activity.

RESULTS

DGKε Inhibition by PA Is Specific for Both Its sn-1 and sn-2 Acyl Chains Contrasting to That of DGKζ—It has previously been shown by our group that the modulation of DGKε by the product of its catalysis, PA, uniquely contrasts to that of other DGKs such as DGKζ; DGKε is inhibited by PA, whereas DGKζ is activated by PA (40). Furthermore, DGKε is the only DGK isoform currently known to possess a selectivity for *sn*-2 arachidonoyl-DAG (36, 39), the main DAG species in the PI turnover cycle (54). Thus, we carried out an investigation to assess the possible role of acyl chain composition in the inhibition of DGKε by PA, another intermediate lipid in the PI turnover cycle. Sf21 expressed DGKε-His_6 was assayed for enzymatic activity using SAG as a lipid substrate in the presence of PAs with varying *sn*-1 and *sn*-2 acyl chains. Similar to the substrate specificity of DGKε for *sn*-2 arachidonoyl-DAG, DGKε was most inhibited by *sn*-2 arachidonoyl-PAs such as 1-palmitoyl-2-arachidonoyl phosphatidic acid (PAPA), 1-stearoyl-2-arachidonoyl phosphatidic acid (SAPA), 1-arachidoyl-2-arachidonoyl phosphatidic acid, or 1,2-diarachidonoyl phosphatidic acid (Fig. 2*A*). For example, when comparing different PAs with *sn*-1 stearoyl acyl chains, SAPA (an *sn*-2 arachidonoyl-PA) showed much greater inhibition of DGKε than other non-*sn*-2 arachidonoyl-PAs such as 1-stearoyl-2-linoleoyl phosphatidic acid (SLPA) or 1-stearoyl-2-oleoyl phosphatidic acid (SOPA) (Fig. 2*A*). In further support of this assertion, when comparing PAs with palmitoyl acyl chains at the *sn*-1 position, PAPA (an *sn*-2 arachidonoyl-PA) showed much greater inhibition than the non-*sn*-2 arachidonoyl-PA 1-palmitoyl-2-oleoyl phosphatidic acid (Fig. 2*B*). Interestingly, when comparing *sn*-2 arachidonoyl-PAs, the PA with the *sn*-1 acyl chain length of 18 carbons (SAPA) showed the greatest inhibition, followed by PAPA with a shorter 16 carbon *sn*-1 acyl chain, then equally by 1-arachidoyl-2-arachidonoyl phosphatidic acid and 1,2-diarachidonoyl phosphatidic acid, each with longer 20 carbon *sn*-1 acyl chains, indicating that DGKε is not only selective for an *sn*-2 arachidonoyl acyl chain but is also selective for the length of the *sn*-1 acyl chain (Fig. 2*A*). Of all the PAs tested, 1-stearoyl-2-arachidonoyl phosphatidic acid (SAPA), a PA with the most prevalent acyl chain composition of lipids involved specifically in the PI turnover cycle (54), was the most potent inhibitor of DGKε (Fig. 2*A*). When the same set of PAs was tested using the less preferred lipid substrate 1-stearoyl-2-linoleoyl-*sn*-glycerol, the qualitative patterns obtained were similar to those obtained using SAG (Fig. 2*C*). SAPA was still a much more effective inhibitor than SLPA (Fig. 2*C*), indicating the results obtained using SAG were valid and independent of lipid substrate. Furthermore, because enriched lysates from Sf21

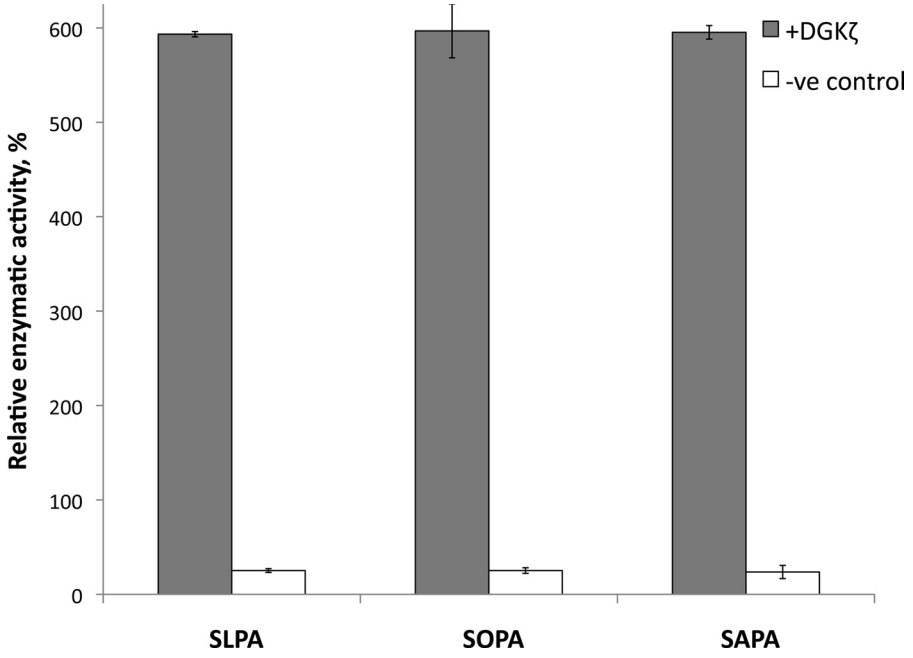


FIGURE 3. **DGKζ is activated by PA in a nonacyl chain-specific manner.** Enriched lysates from Sf21-overexpressing DGKζ-FLAG (+*DGKζ*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity with 7.5 mM Triton X-100, 7.5 mM Triton X-114, 0.1 mM [γ-^{32}P]ATP, and 1.5 mol % DOG, in the presence of either 8.1 mol % SLPA, SOPA, or SAPA. The enzymatic activity presented was normalized to DGKζ enzymatic activity in the absence of PA, which was 0.252 ± 0.001 nmol of PA/min and defined as 100% enzymatic activity.

cells overexpressing DGKε-His$_6$ were used (throughout this study) to assay for DGKε-His$_6$ enzymatic activity, it was confirmed that specific DGKε-His$_6$ enzymatic activity was being measured rather than enzymatic activity from another enzyme found in Sf21 cells; DGK enzymatic activity of enriched lysates from Sf21 cells overexpressing DGKε-His$_6$ was at least 13 times higher than the DGK enzymatic activity of enriched lysates from mock-infected Sf21 cells using either SAG or 1-stearoyl-2-linoleoyl-*sn*-glycerol as a DAG lipid substrate (depending on the PA used) (Fig. 2).

In contrast to DGKε, when different *sn*-1 stearoyl-PAs were tested for their effect on Sf21-expressed DGKζ-FLAG (DGKζ being a DGK isoform not known to be involved in the PI turnover cycle) (Fig. 1), its enzymatic activity was increased in the presence of these PAs, as was shown previously (40), and were all within the error of each other (Fig. 3). The effects of different PAs on DGKζ were compared using 8 mol % PA to make the comparison between the different PAs potentially more sensitive to differences in enzymatic activity. When we used the same PAs at a concentration of 2 mol % to assess their effect on DGKζ, the qualitative patterns of DGKζ activation by the different PAs were the same as when using 8 mol % PA; the increases in the enzymatic activity of DGKζ by the different PAs were all within error of each other, although this increase in enzymatic activity was less pronounced (data not shown). These results make it unlikely that the differences among the different PAs in the inhibition of DGKε were due to varying impurities or concentrations of the various PA lipids. In addition, because enriched lysates from Sf21 cells overexpressing DGKζ-FLAG were used (throughout this study) to assay for DGKζ-FLAG enzymatic activity, it was confirmed that specific DGKζ-FLAG enzymatic activity was being measured rather than enzymatic activity from another enzyme found in Sf21 cells. The DGK enzymatic activity detected in the enriched lysates from Sf21 cells overexpressing DGKζ-FLAG was at least 23 times higher than the DGK enzymatic activity of enriched lysates from mock-infected Sf21 cells (depending on the PA used) (Fig. 3). Taken together, these results show that inhibition of DGKε is specific for both the *sn*-1 and *sn*-2 acyl chains of PA that contrasts to the nonspecific activation of DGKζ by PA of different acyl chain compositions.

DGKε Substrate Specificity Is Based upon a Combined Selectivity toward the sn-1 and sn-2 Acyl Chain of DAG—Although the substrate specificity of DGKε for DAG with an *sn*-2 arachidonoyl acyl chain has been well established (36), its selectivity for the *sn*-1 acyl chain has not been investigated. Thus, we carried out experiments to assess the selectivity of DGKε for the *sn*-1 acyl chain of DAG. When Sf21-expressed DGKε-His$_6$ was assayed for enzymatic activity using *sn*-2 arachidonoyl-DAGs with increasing lengths of the saturated *sn*-1 acyl chain, the DAG with an intermediate 18 carbon *sn*-1 acyl chain length (SAG) was phosphorylated at the highest rate by DGKε, followed by the shorter 16 carbon *sn*-1 acyl chain in PAG and then followed by the longer 20-carbon *sn*-1 acyl chain in 1-arachidoyl-2-arachidonoyl-*sn*-glycerol (AAG) (Fig. 4). Thus, similar to DGKε inhibition by PA, in terms of its substrate specificity, DGKε was selective for the length of the *sn*-1 acyl chain of DAG. As established before (36), DGKε catalyzed the phosphorylation of 1,2-dioleoyl-*sn*-glycerol at a rate much lower than the *sn*-2 arachidonoyl-DAGs, confirming the selectivity of DGKε for the *sn*-2 arachidonoyl acyl chain (Fig. 4). Furthermore, of all DAGs tested, DGKε was most selective for DAG with the most abundant acyl chain composition of lipids involved specifically in the PI turnover cycle (54), 1-stearoyl-2-arachidonoyl, which matches the selectivity of DGKε for PA with the 1-stearoyl-2-arachidonoyl acyl chain composition (Fig. 4). When Sf21-expressed DGKζ-FLAG was assayed using the same set of DAGs, the rates of phosphorylation of these DAGs were within error of each other (Fig. 5), providing a contrasting picture to the DAG acyl chain selectivity of DGKε and making it unlikely that the differences among the different DAGs in terms of the rate of their phosphorylation by DGKε were due to varying impurities or concentrations of the various DAG lipids. In addition, the DGK enzymatic activity detected in the enriched lysates from Sf21 cells overexpressing DGKε-His$_6$ or DGKζ-FLAG was at least 3.5 or 32 times higher, respectively,

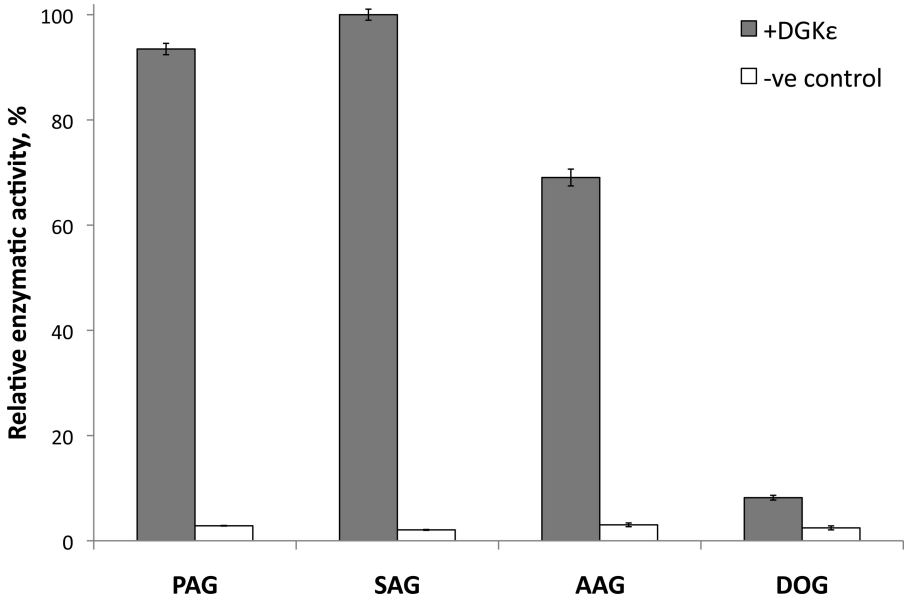


FIGURE 4. **DGKε substrate specificity is dependent upon both the *sn*-1 and *sn*-2 acyl chains of DAG.** Enriched lysates from Sf21-overexpressing DGKε-His_6 (+*DGKε*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity with 7.5 mM Triton X-100, 7.5 mM Triton X-114, and 0.1 mM [γ-^{32}P]ATP, using either 0.38 mol % PAG, SAG, AAG, or DOG as a DAG lipid substrate. The enzymatic activity presented was normalized to DGKε enzymatic activity using SAG as a DAG lipid substrate, which was 0.234 ± 0.002 nmol of PA/min and defined as 100% enzymatic activity.

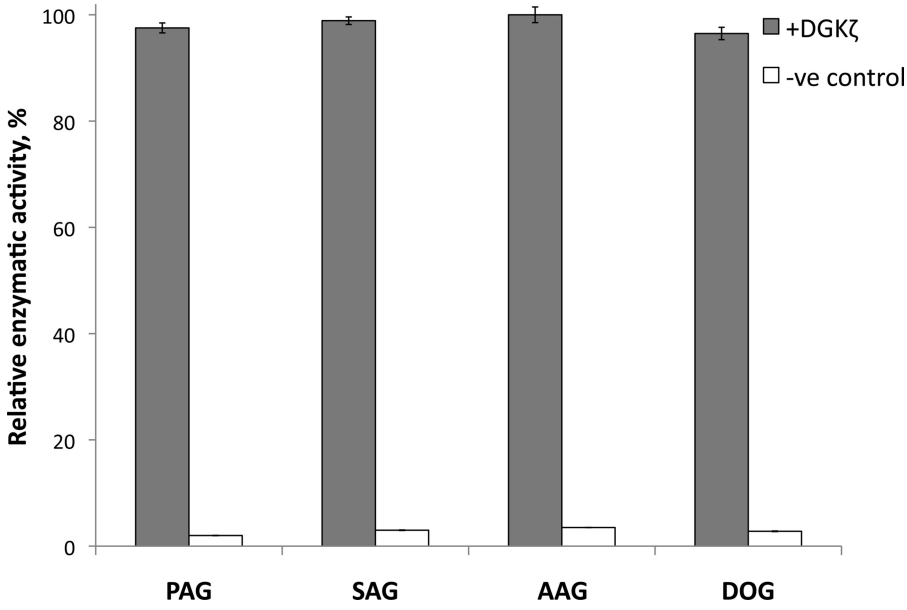


FIGURE 5. **DGKζ does not exhibit an acyl chain-dependent substrate specificity.** Enriched lysates from Sf21-overexpressing DGKζ-FLAG (+*DGKζ*) or from mock baculovirus-infected Sf21 cells (−*ve control*) were assayed for DGK enzymatic activity with 7.5 mM Triton X-100, 7.5 mM Triton X-114, and 0.1 mM [γ-^{32}P]ATP, using either 0.38 mol % PAG, SAG, AAG, or DOG as a DAG lipid substrate. The enzymatic activity presented was normalized to DGKζ enzymatic activity using AAG as a DAG lipid substrate, which was 0.169 ± 0.025 nmol of PA/min and defined as 100% enzymatic activity.

than the DGK enzymatic activity of enriched lysates from mock-infected Sf21 cells (depending on the DAG used) (Figs. 4 and 5), consistent with previous results presented in this study (Figs. 2 and 3) and demonstrating that specific DGKζ-FLAG enzymatic activity was being assayed. Therefore, taken together, these sets of experiments show that DGKε substrate specificity is based upon a selectivity for the *sn*-1 acyl chain of DAG, as well as the *sn*-2 acyl chain.

Lipidomic Analysis of DGKε KO and WT MEFs Supports DGKε Selectivity for DAG with a 1-Stearoyl-2-arachidonoyl Acyl Chain Composition—With *in vitro* experiments showing the selectivity of DGKε for DAG with a 1-stearoyl-2-arachidonoyl acyl chain composition, we performed a lipidomic analysis on DGKε KO and WT MEFs to provide *in vivo* evidence for this acyl chain selectivity. Prior research comparing DGKε KO MEFs with WT has demonstrated a profoundly different behavior in 20:4 (arachidonoyl)-containing PI lipids relative to other major PI species, particularly 36:2 PI (44), supporting the well known selectivity of DGKε for the *sn*-2 arachidonoyl acyl chain of DAG. Using the same data to examine changes in 18:0 (stearoyl)-containing lipids of all glycerophospholipid classes, we found that there was 29% less 18:0 (stearoyl)-containing PI in DGKε KO than the WT samples ($p < 0.01$), supporting selectivity of DGKε for an *sn*-1 stearoyl acyl chain of DAG as shown by *in vitro* experiments in this study (Fig. 6*A*). Thus, the pool of the 18:0 (stearoyl)-containing PI (34:1, 36:1, 36:3, 38:2, 38:3, 38:4, and 40:5) behaves similarly to 20:4 (arachidonoyl)-containing PI with respect to genotype. Note that the large mass of 38:4 PI dominates both groupings, supporting the combined selectivity of DGKε for DAG with a 1-stearoyl-2-arachidonoyl acyl chain composition (Fig. 6*A*). There was also no significant difference for the 16:0 (palmitoyl)-containing lipids of any class other than PI, which showed slightly less 16:0-containing PI in the KO cells (Fig. 6*B*). Thus, despite the similarity between 16:0 and 18:0 acyl chains, deletion of DGKε results in a larger decrease in 18:0 compared with 16:0 PI. Additionally, several species of di-18:1 (oleoyl) lipids (36:2 PA, PC, and phosphatidylethanolamine) showed substantial increases in the knock-out cells (28% $p = 0.012$, 38% $p < 0.01$, and 26% $p < 0.01$, respectively). However, there was no significant difference in 36:2 PI between the WT and KO cells (Fig. 6*C*). Taken together, these results provide *in vivo* evidence of the selectivity of DGKε for DAG with a 1-stearoyl-2-arachidonoyl acyl chain composition through the enrichment of PI with this acyl chain composition.

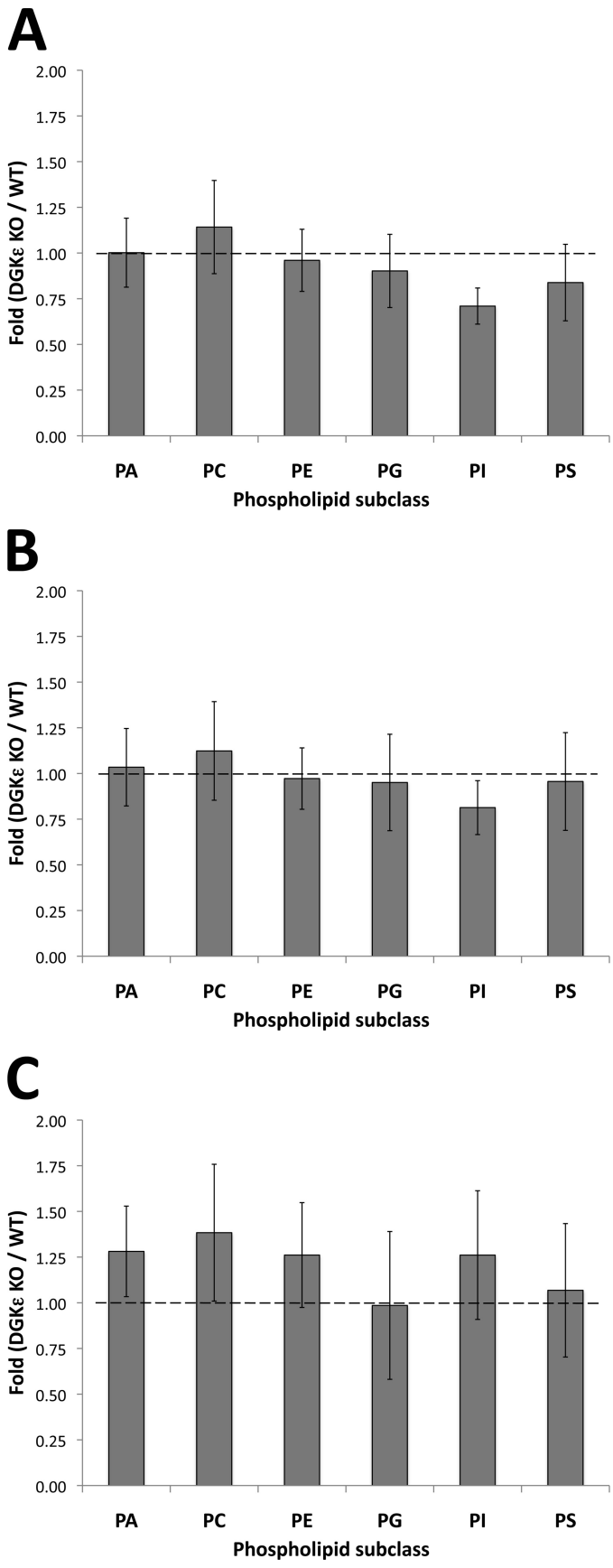


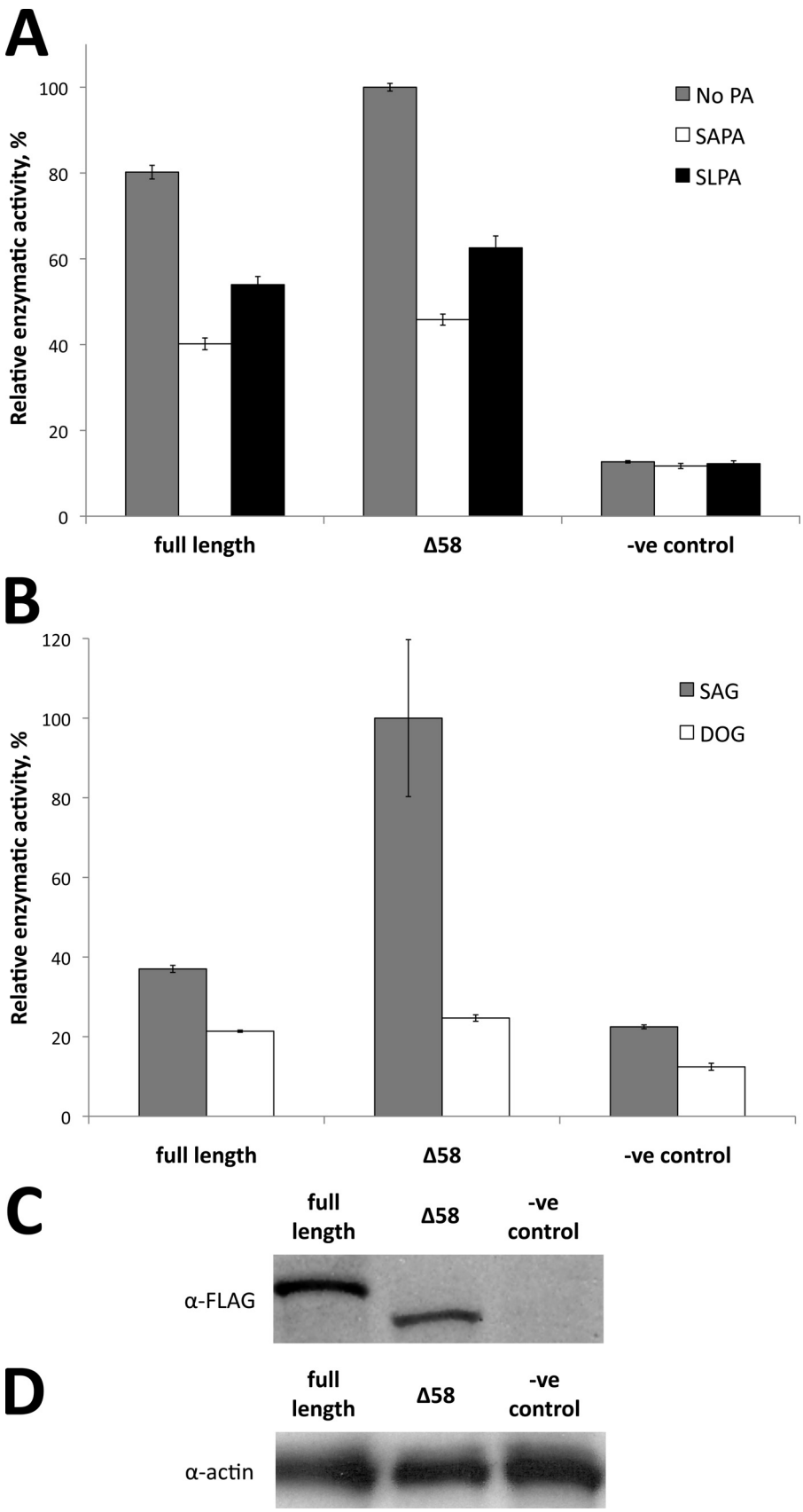
FIGURE 6. **Lipidomic analysis of DGKε KO and WT MEFs.** Phospholipids in DGKε KO and WT MEFs were determined by direct infusion mass spectrometry and identified by liquid chromatography-tandem mass spectrometry. Different phospholipid classes (*PA*, phosphatidic acids; *PC*, phosphatidylcholines; *PE*, phosphatidylethanolamines; *PG*, phosphatidylglycerols; *PI*, phosphatidylinositols; *PS*, phosphatidylserines) within a specific group of phospholipids with the same acyl chain composition were compared. Fold differences in the KO samples compared with the WT samples are presented as well as the standard deviation from three independent experiments (each in triplicate). *A*, 18:0-containing phospholipids; *B*, 16:0-containing phospholipids; *C*, 36:2 phospholipids.

Deletion of the Hydrophobic Segment and Cationic Cluster from DGKε Retains Its PA or DAG Acyl Chain Selectivity—The PA acyl chain-specific inhibition of DGKε may possibly be the result of the binding of the cationic cluster and hydrophobic segment of DGKε to the anionic headgroup and acyl chains of PA, respectively. Thus, to test the role of the cationic cluster and hydrophobic segment in the PA inhibition of DGKε, a 58-amino acid N-terminal truncation of DGKε lacking its cationic cluster and hydrophobic segment (3XFLAG-DGKεΔ58) was used (Fig. 1). When enriched lysates from COS-7 cells overexpressing 3XFLAG-DGKεΔ58 or 3XFLAG-DGKε full-length (3XFLAG-DGKεFL) proteins were assayed for DGK enzymatic activity in the presence of SAPA or SLPA, both 3XFLAG-DGKεFL and 3XFLAG-DGKεΔ58 showed significant inhibition in the presence of these PAs when compared with their enzymatic activity in the absence of these PAs (Fig. 7*A*). Furthermore, there was less inhibition of both the 3XFLAG-DGKεFL and 3XFLAG-DGKεΔ58 in the presence of SLPA compared with SAPA (Fig. 7*A*), corresponding to data obtained with Sf21-expressed DGKε-His_6 (Fig. 2*A*). In support of a possible common mechanism of PA or DAG acyl chain selectivity, 3XFLAG-DGKεΔ58 showed the same substrate selectivity for SAG over DOG as compared with 3XFLAG-DGKεFL (Fig. 7*B*), supporting previous published results from our group, which showed that the deletion of the hydrophobic segment alone does not affect DGKε substrate selectivity (38).

Validating the measurement of specific 3XFLAG-DGKεFL or 3XFLAG-DGKεΔ58 enzymatic activity, the DGK enzymatic activity of enriched lysates from COS-7 cells overexpressing 3XFLAG-DGKεFL or 3XFLAG-DGKεΔ58 was at least 1.5 times higher (depending on which DGK construct and PA was used) than the DGK enzymatic activity detected in the enriched lysates from COS-7 cells transfected with p3XFLAG-CMV-7.1 empty vector (Fig. 7, *A* and *B*). Furthermore, an immunoblot performed on enriched lysates from these COS-7 cells overexpressing 3XFLAG-DGKεΔ58 or 3XFLAG-DGKεFL proteins after extraction with the detergent Nonidet P-40 showed the detection of one 60- or 67-kDa band, respectively, matching their predicted molecular mass (Fig. 7*C*); no band was present in enriched lysates from COS-7 cells transfected with the p3XFLAG-CMV-7.1 empty vector (Fig. 7*C*). In addition, all samples showed an equal amount of actin by immunoblot analysis, indicating that the lower DGK enzymatic activity in enriched lysates from COS-7 cells transfected with empty vector is not due to decreased amount of lysate assayed (Fig. 7*D*). Therefore, taken together, these results rule out the cationic cluster and hydrophobic segment as domains responsible for the PA acyl chain-dependent inhibition of DGKε and consequently rule out the involvement of these regions in acyl chain selectivity of DGKε.

DGKε Inhibition by PA Is Competitive—The similarity of the selectivity of DGKε for both the *sn*-1 and *sn*-2 acyl chains of either PA or DAG as well as the elimination of the cationic cluster/hydrophobic segment as domains responsible for acyl chain-specific PA inhibition and substrate specificity of DGKε as shown in this study suggest that PA could possibly be competing with DAG for the active site of DGKε. With these results in mind, we performed an analysis of the kinetics of DGKε PA inhibition to determine the type of enzyme inhibition. Thus, Sf21-expressed DGKε-His_6 was assayed for enzymatic activity at varying concentrations of the DAG lipid substrate SAG in the presence of differing concentrations of the PA inhibitor SAPA ranging from 0 to 3.40 mol %. For these sets of experiments, OG/DOPC-based mixed micelles were used instead of the Triton X-100/Triton X-114/DOPC-based mixed micelles used in the other experiments in this study because of deviations from Michaelis-Menten kinetics using Triton X-100/Triton X-114/DOPC-based mixed micelles (data not shown). Nevertheless, we confirmed that when using OG/DOPC-based mixed micelles, DGKε showed the same qualitative patterns of PA acyl chain-specific inhibition (Fig. 8) as with the Triton X-100/Triton X-114/DOPC-based mixed micelles (Fig. 2*A*). Consistent with previous results presented in this study (Fig. 2*A*), DGK enzymatic activity of enriched lysates from Sf21 cells overexpressing DGKε-His_6 was at least 13 times higher (depending on which PA was used) than the DGK enzymatic activity detected in the enriched lysates from mock-infected Sf21 cells (Fig. 8), demonstrating that specific DGKε-His_6 enzymatic activity was being measured.

When the kinetic data of DGKε inhibition by PA was analyzed using a Cornish-Bowden plot ((substrate concentration) (initial velocity)$^{-1}$ *versus* inhibitor concentration) (55), the linear regression lines for data



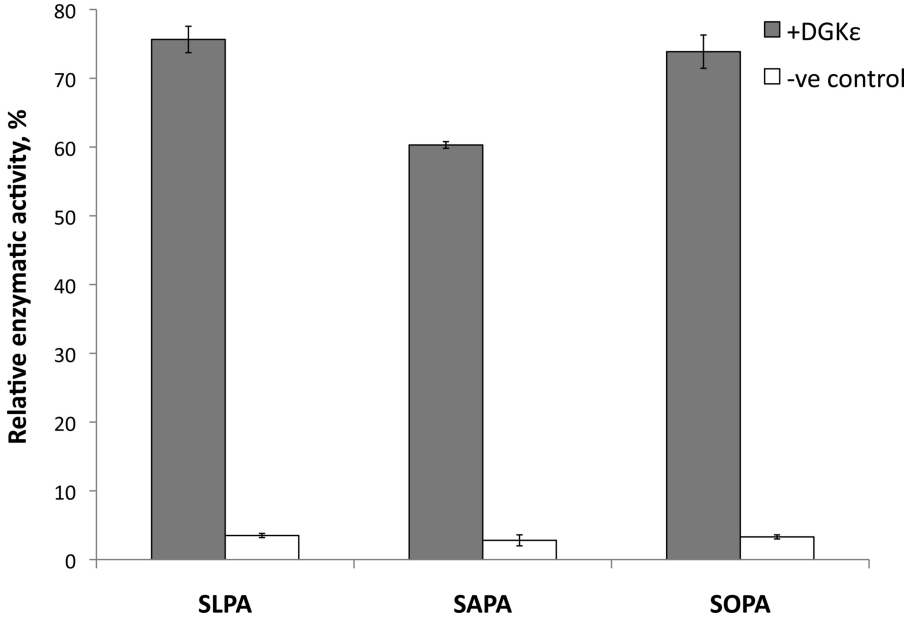


FIGURE 8. **DGKε acyl chain-specific inhibition by PA is detectable using OG/DOPC-based mixed micelles.** Enriched lysates from Sf21-overexpressing DGKε-His_6 (*+DGKε*) or from mock baculovirus-infected Sf21 cells (*−ve control*) were assayed for DGK enzymatic activity with 75 mM OG, 0.1 mM [γ-^{32}P]ATP, and 2.0 mol % SAG, in the presence of either 2.0 mol % SLPA, SAPA, or SOPA. The enzymatic activity presented was normalized to DGKε enzymatic activity in the absence of PA, which was 1.056 ± 0.043 nmol of PA/min and defined as 100% enzymatic activity.

plotted for each concentration of SAG were parallel and not significantly different from each other with a pooled slope value of 0.12 ± 0.02 (mol % SAG) (mol % SAPA)$^{-1}$ (nmol PA)$^{-1}$ (min), most closely approximating a competitive type of inhibition (Fig. 9). This competitive inhibition was confirmed by nonlinear regression analysis of the kinetic data, which had the highest degree of fit to a nonlinear expression for a competitive type of enzyme inhibition as determined by possessing the highest coefficient of determination and the lowest Akaike information criterion value. In addition, this nonlinear regression analysis (fitted to a competitive inhibition model) was used to determine the K_i and K_m values; these values were determined to be 2.39 ± 0.26 mol % SAPA and 1.0 ± 0.10 mol % SAG, respectively. A competitive inhibition in which both PA and DAG are competing for the same active site is further confirmed by data from this study showing the same qualitative pattern of selectivity for both acyl chains of its substrate or product, selecting for an *sn*-2 arachidonoyl acyl chain as well as an intermediate length (18 carbon) *sn*-1 acyl chain (Figs. 2 and 4). Interestingly, the kinetics of PA inhibition of DGKε shown in this work differs markedly from that reported by Walsh *et al.* (49) for the PA inhibition of arachidonoyl-specific DGK from bovine testes. The PA inhibition of this arachidonoyl-specific DGK from bovine testes was observed to be noncompetitive with SAG but competitive with ATP, indicating a different mechanism of inhibition and supporting the suggestion that this arachidonoyl-specific DGK is distinct from DGKε. This arachidonoyl-specific DGK also has a different mass of 58 kDa compared with 64 kDa for DGKε (39). Thus, it could be a different splice variant of DGKε.

DISCUSSION

DGKε is currently the only mammalian DGK isoform inhibited by PA, an anionic phospholipid that is the product of the reaction catalyzed by DGKε. Furthermore, DGKε is unique among the mammalian DGK isoforms in that it shows substrate specificity for *sn*-2 arachidonoyl-DAG, the most abundant form of DAG in the PI turnover cycle (54). Here, we show that DGKε inhibition by PA and phosphorylation of DAG are specific for both the *sn*-1 and *sn*-2 acyl chains of PA or DAG, respectively (Figs. 2 and 4). This selectivity for the acyl chains of PA or DAG culminates in a selectivity for a stearoyl *sn*-1 and an arachidonoyl *sn*-2 acyl chain (Figs. 2 and 4), the most prevalent acyl chain composition of lipids involved specifically in the PI turnover cycle (54). Lipidomic analysis of DGKε KO and WT MEFs showing a reduction in stearoyl and arachidonoyl content of PI in DGKε KO MEFs provides *in vivo* evidence for dual acyl chain selectivity of DGKε; the loss of DGKε in the KO MEFs presumably results in the loss of PI enrichment with stearoyl and arachidonoyl acyl chains. Because PI is neither a substrate nor a product of the reaction catalyzed by DGKε, these results dramatically demonstrate the importance of DGKε in the PI turnover cycle. These results obtained with DGKε also directly contrast that of DGKζ, a DGK isoform that is representative of other DGK isoforms in terms of its activation by PA and the lack of DAG acyl chain selectivity. Indeed, DGKζ did not exhibit an acyl chain-specific activation by PA and did not exhibit a selectivity for the acyl chains of DAG (Figs. 3 and 5), suggesting that DGKζ and other isoforms play a very different role in the cell compared with DGKε.

Although DGKε phosphorylates SAG more rapidly than PAG (Fig. 4), the difference is small. Nevertheless, there is a substantial enrichment of PI with 18:0 lipids compared with

FIGURE 7. **DGKε PA or DAG acyl chain selectivity is retained after deletion of the cationic cluster and hydrophobic segment of DGKε.** COS-7 cells overexpressing 3XFLAG-DGKεFL (full-length) or 3XFLAG-DGKεΔ58 (Δ58) protein or transfected with empty vector (*−ve control*) were resuspended in lysis buffer containing 1% Nonidet P-40 and centrifuged at 100 000 × *g* for 30 min. *A,* resultant supernatants (enriched lysates) were assayed for DGK enzymatic activity with 7.5 mM Triton X-100, 7.5 mM Triton X-114, 0.1 mM [γ-^{32}P]ATP, and 3.0 mol % SAG, in the presence of either 1.5 mol % SAPA or SLPA. These enzymatic activity values are presented as a percentage of 3XFLAG-DGKεΔ58 enzymatic activity in the absence of PA, which was 0.038 ± 0.003 nmol of PA/min. *B,* enriched lysates were also assayed for DGK enzymatic activity with 75 mM OG and 0.5 mM [γ-^{32}P]ATP, using either 0.30 mol % SAG or DOG as a DAG lipid substrate. These enzymatic activity values are presented as a percentage of 3XFLAG-DGKεΔ58 enzymatic activity using SAG as a lipid substrate, which was 0.0054 ± 0.0011 nmol of PA/min. An immunoblot was also performed on the enriched lysates using a 1:2500 dilution of mouse anti-FLAG M2 primary, 1:2500 dilution of goat anti-mouse horseradish peroxidase secondary antibody (*C*) or a 1:500 dilution of goat anti-actin primary, 1:2500 dilution of donkey anti-goat horseradish peroxidase secondary antibody (*D*).

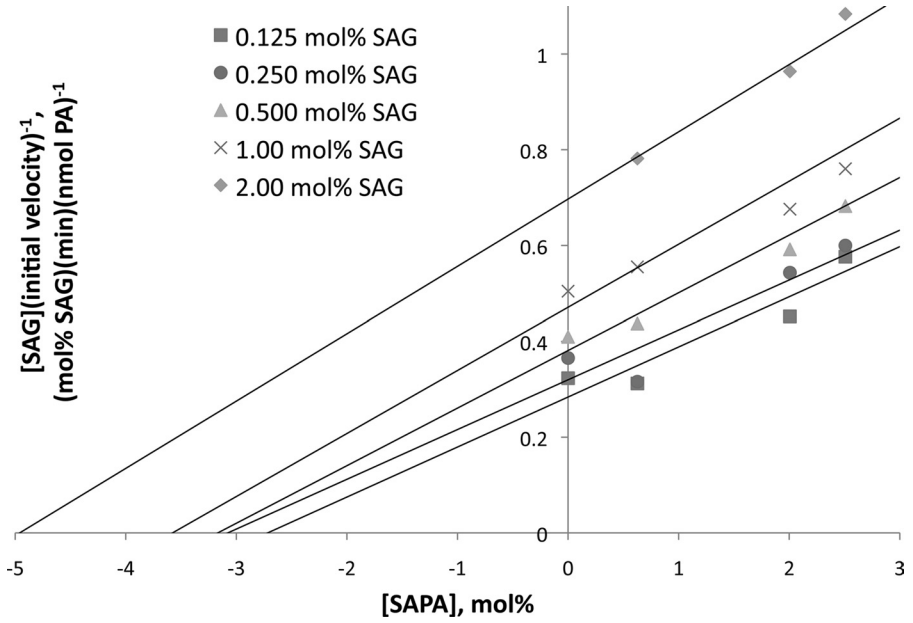


FIGURE 9. **Cornish-Bowden plot for DGKε phosphorylation of SAG in the presence of different concentrations of SAPA indicates that the inhibition of DGKε by PA is competitive.** Enriched lysates from Sf21-overexpressing DGKε-His_6 were assayed for DGK enzymatic activity assayed for enzymatic activity with 75 mM OG, 0.1 mM [γ-^{32}P]ATP, either 0.125, 0.250, 0.500, 1.00, or 2.00 mol % SAG, and either 0, 0.630, 2.00, 2.50, or 3.40 mol % SAPA.

other *sn-1* acyl chains (Fig. 6). There are several possible factors responsible for this amplification. One factor is that the reaction catalyzed by DGKε is a step in the PI turnover cycle. PA is converted to PI and then to DAG followed by the conversion of DAG to PA catalyzed by DGKε. Each time this cycle goes around once, there is a small enrichment of the lipid intermediates of this cycle with 1-stearoyl-2-arachidonoyl acyl chains. Through multiple iterations of this cycle resulting in the regeneration of the lipid intermediates, there can be a substantial enrichment of these lipid intermediates with specific acyl chains, despite the modest selectivity of DGKε in a single reaction. In addition to this factor, some of the lipid intermediates in this cycle also affect the activity of other enzymes in this cycle. If this modulation of enzyme activity exhibits acyl chain specificity, it can also contribute to acyl chain enrichment as can modulation of the rate of lipid transfer between the plasma membrane and endoplasmic reticulum.

The selectivity of DGKε for the same acyl chain composition in PA (Fig. 2) or DAG (Fig. 4) taken together with our results indicating that the PA inhibition of DGKε is competitive (Fig. 9) suggests that PA binds and competes with DAG for the same active/binding site of DGKε. This postulation is supported by our findings showing that the deletion of the cationic cluster/hydrophobic segment of DGKε has no effect on its PA (Fig. 7*A*) or DAG (Fig. 7*B*) acyl chain selectivity, further eliminating these regions as candidates for the active site of DGKε. However, the specific region within DGKε that encompasses this active site is a question that remains unclear. In any case, this active/binding site for PA or DAG is the only known DGK that not only recognizes the lipid headgroup, as is common with many lipid recognition domains (56), but as shown in this study simultaneously recognizes the length and saturation of the two lipid acyl chains. This degree of specificity of an enzyme acting on, or affected by, lipids is indeed unusual.

The implications of the results shown in this study in terms of the role and the regulation of DGKε in cell signaling are quite intriguing. DGKε is the only DGK isoform that has been proposed to be fully committed to the PI turnover cycle in the enrichment of PI with arachidonate through its selectivity for *sn*-2 arachidonoyl-DAG (36), a hypothesis supported by *in vivo* data (44, 57). Furthermore, DGKε is the only DGK isoform that is constitutively active at the membrane and is inhibited by PA (an anionic phospholipid involved in the PI turnover cycle) (40). By contrast, all other DGK isoforms are thought to be sequestered from their substrate in the cytoplasm (or nucleoplasm) and engage DAG only by translocating to a membrane compartment (2, 4). Furthermore, all other DGK isoforms examined to date are activated by PA (40, 48, 58, 59). Thus, DGKε inhibition by PA, specifically a form of PA that is most abundant in the PI turnover cycle (1-stearoyl-2-arachidonoyl phosphatidic acid), could serve as an additional mechanism used by DGKε to fully commit itself to the PI turnover cycle by acting as a feedback mechanism to negatively regulate the constitutively active enzymatic activity of DGKε in a PI turnover cycle-specific manner. This inhibition would be PI turnover cycle-specific because other PAs with other acyl chain compositions not abundant in this pathway, formed through other DGK isoforms or phospholipase D cleavage of PC, would have a smaller effect on DGKε enzymatic activity. Because DGKε catalyzes the first step in the recycling of arachidonoyl-DAG to PtdIns(4,5)P_2 via PA, an unregulated constitutively active DGKε would lead to the unintended accumulation of many PI turnover cycle lipids; many of these lipids such as PA and PtdIns(4,5)P_2 are potent signaling molecules in the cell. A disruption in the balance of these potent signaling molecules in the cell would have drastic effects on various cellular functions. This mechanism of acyl chain selectivity in the inhibition of DGKε can be applied to other PI turnover cycle lipids such as PtdIns(4,5)P_2, an anionic phospholipid that has been shown to inhibit DGKε (40).

In conclusion, the results in this study show that DGKε possesses selectivity for a combination of the *sn*-1 and *sn*-2 acyl chains in either PA or DAG in a mechanism that suggests that PA competes with DAG for the active site of DGKε. These results bring forward a unique and novel mechanism of DGKε regulation in the PI turnover cycle where its dual acyl chain selectivity for the most prevalent acyl chain composition in this cycle is used to negatively regulate its enzymatic activity in a manner that ensures that DGKε remains fully committed to the PI turnover cycle. This novel mechanism of enzyme regulation within a signaling pathway could serve as a template for regulation of enzymes in other cellular pathways.

REFERENCES

1. Goto, K., and Kondo, H. (1999) *Chem. Phys. Lipids* **98,** 109–117
2. van Blitterswijk, W. J., and House, B. (2000) *Cell. Signal.* **12,** 595–605
3. Kanoh, H., Yamada, K., and Sakane, F. (2002) *J. Biochem.* **131,** 629–633
4. Topham, M. K. (2006) *J. Cell. Biochem.* **97,** 474–484
5. Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007) *Biochim. Biophys. Acta* **1771,** 793–806
6. Goto, K., Hozumi, Y., Nakano, T., Saino-Saito, S., and Martelli, A. M. (2008) *Tohoku J. Exp. Med.* **214,** 199–212
7. Mérida, I., Avila-Flores, A., and Merino, E. (2008) *Biochem. J.* **409,** 1–18
8. Topham, M. K., and Epand, R. M. (2009) *Biochim. Biophys. Acta* **1790,** 416–424
9. Carrasco, S., and Mérida, I. (2007) *Trends Biochem. Sci.* **32,** 27–36
10. Wakelam, M. J. (1998) *Biochim. Biophys. Acta* **1436,** 117–126
11. Ron, D., and Kazanietz, M. G. (1999) *FASEB J.* **13,** 1658–1676
12. Newton, A. C. (1997) *Curr. Opin. Cell Biol.* **9,** 161–167
13. Toker, A. (1998) *Front. Biosci.* **3,** D1134–D1147
14. Parekh, D. B., Ziegler, W., and Parker, P. J. (2000) *EMBO J.* **19,** 496–503
15. Lucas, P., Ukhanov, K., Leinders-Zufall, T., and Zufall, F. (2003) *Neuron* **40,** 551–561
16. Caloca, M. J., Garcia-Bermejo, M. L., Blumberg, P. M., Lewin, N. E., Kremmer, E., Mischak, H., Wang, S., Nacro, K., Bienfait, B., Marquez, V. E., and Kazanietz, M. G. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11854–11859
17. Jenkins, G. H., Fisette, P. L., and Anderson, R. A. (1994) *J. Biol. Chem.* **269,** 11547–11554
18. Jones, G. A., and Carpenter, G. (1993) *J. Biol. Chem.* **268,** 20845–20850
19. Limatola, C., Schaap, D., Moolenaar, W. H., and van Blitterswijk, W. J. (1994) *Biochem. J.* **304,** 1001–1008
20. Jones, J. A., and Hannun, Y. A. (2002) *J. Biol. Chem.* **277,** 15530–15538
21. Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) *Science* **294,** 1942–1945
22. Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S., and Ktistakis, N. T. (2004) *J. Biol. Chem.* **279,** 44763–44774
23. Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996) *J. Biol. Chem.* **271,** 8472–8480
24. Siddhanta, A., and Shields, D. (1998) *J. Biol. Chem.* **273,** 17995–17998
25. van Corven, E. J., van Rijswijk, A., Jalink, K., van der Bend, R. L., van Blitterswijk, W. J., and Moolenaar, W. H. (1992) *Biochem. J.* **281,** 163–169
26. Han, G. S., O'Hara, L., Carman, G. M., and Siniossoglou, S. (2008) *J. Biol. Chem.* **283,** 20433–20442
27. Han, G. S., O'Hara, L., Siniossoglou, S., and Carman, G. M. (2008) *J. Biol. Chem.* **283,** 20443–20453
28. Harden, N., Yap, S. F., Chiam, M. A., and Lim, L. (1993) *Biochem. J.* **289,** 439–444
29. Masai, I., Hosoya, T., Kojima, S., and Hotta, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6030–6034
30. Masai, I., Okazaki, A., Hosoya, T., and Hotta, Y. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11157–11161
31. De La Roche, M. A., Smith, J. L., Rico, M., Carrasco, S., Merida, I., Licate, L., Côté, G. P., and Egelhoff, T. T. (2002) *Biochem. J.* **368,** 809–815
32. Miller, K. G., Emerson, M. D., and Rand, J. B. (1999) *Neuron* **24,** 323–333
33. Katagiri, T., Mizoguchi, T., and Shinozaki, K. (1996) *Plant Mol. Biol.* **30,** 647–653
34. Gómez-Merino, F. C., Brearley, C. A., Ornatowska, M., Abdel-Haliem, M. E., Zanor, M. I., and Mueller-Roeber, B. (2004) *J. Biol. Chem.* **279,** 8230–8241
35. Gómez-Merino, F. C., Arana-Ceballos, F. A., Trejo-Téllez, L. I., Skirycz, A., Brearley, C. A., Dörmann, P., and Mueller-Roeber, B. (2005) *J. Biol. Chem.* **280,** 34888–34899
36. Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) *J. Biol. Chem.* **271,** 10237–10241
37. Decaffmeyer, M., Shulga, Y. V., Dicu, A. O., Thomas, A., Truant, R., Topham, M. K., Brasseur, R., and Epand, R. M. (2008) *J. Mol. Biol.* **383,** 797–809
38. Dicu, A. O., Topham, M. K., Ottaway, L., and Epand, R. M. (2007) *Biochemistry* **46,** 6109–6117
39. Walsh, J. P., Suen, R., Lemaitre, R. N., and Glomset, J. A. (1994) *J. Biol. Chem.* **269,** 21155–21164
40. Thirugnanam, S., Topham, M. K., and Epand, R. M. (2001) *Biochemistry* **40,** 10607–10613
41. Epand, R. M., Shulga, Y. V., Timmons, H. C., Perri, A. L., Belani, J. D., Perinpanathan, K., Johnson-McIntire, L. B., Bajjalieh, S., Dicu, A. O., Elias, C., Rychnovsky, S. D., and Topham, M. K. (2007) *Biochemistry* **46,** 14225–14231
42. Postle, A. D., Dombrowsky, H., Clarke, H., Pynn, C. J., Koster, G., and Hunt, A. N. (2004) *Biochem. Soc. Trans.* **32,** 1057–1059
43. Toker, A. (2002) *Cell. Mol. Life Sci.* **59,** 761–779
44. Milne, S. B., Ivanova, P. T., Armstrong, M. D., Myers, D. S., Lubarda, J., Shulga, Y. V., Topham, M. K., Brown, H. A., and Epand, R. M. (2008) *Biochemistry* **47,** 9372–9379
45. Pettitt, T. R., and Wakelam, M. J. (1999) *J. Biol. Chem.* **274,** 36181–36186
46. Sakane, F., Yamada, K., Imai, S., and Kanoh, H. (1991) *J. Biol. Chem.* **266,** 7096–7100
47. Kai, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994) *J. Biol. Chem.* **269,** 18492–18498
48. Tu-Sekine, B., Ostroski, M., and Raben, D. M. (2007) *Biochemistry* **46,** 924–932
49. Walsh, J. P., Suen, R., and Glomset, J. A. (1995) *J. Biol. Chem.* **270,** 28647–28653
50. Ames, B. N. (1966) *Methods Enzymol.* **8,** 115–118
51. Carman, G. M., Deems, R. A., and Dennis, E. A. (1995) *J. Biol. Chem.* **270,** 18711–18714
52. Deems, R. A. (2000) *Anal. Biochem.* **287,** 1–16
53. Ivanova, P. T., Milne, S. B., Byrne, M. O., Xiang, Y., and Brown, H. A. (2007) *Methods Enzymol.* **432,** 21–57
54. Holub, B. J., and Kuksis, A. (1978) *Adv. Lipid Res.* **16,** 1–125
55. Cornish-Bowden, A. (1974) *Biochem. J.* **137,** 143–144
56. Lemmon, M. A. (2008) *Nat. Rev. Mol. Cell Biol.* **9,** 99–111
57. Rodriguez de Turco, E. B., Tang, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., Taketomi, A., Prescott, S. M., and Bazan, N. G. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4740–4745
58. Ostroski, M., Tu-Sekine, B., and Raben, D. M. (2005) *Biochemistry* **44,** 10199–10207
59. Ciprés, A., Carrasco, S., Merino, E., Díaz, E., Krishna, U. M., Falck, J. R., Martínez-A, C., and Mérida, I. (2003) *J. Biol. Chem.* **278,** 35629–35635