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STUDY OF ARACHIDONOYL SPECIFICITY IN TWO ENZYMES OF THE PI-CYCLE

Yulia V. Shulga¹, Matthew K. Topham², and Richard M. Epand¹

¹ Department of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, CANADA

² Huntsman Cancer Institute, University of Utah, 2000 Circle of Hope, Salt Lake City, Utah 84112, U.S.A

Abstract

We identified a conserved pattern of residues L-X_(3–4)-R-X₍₂₎-L-X₍₄₎-G, in which -X_(n)- represents n residues of any amino acids, in two enzymes acting on polyunsaturated fatty acids, diacylglycerol kinase epsilon (DGKε) and phosphatidylinositol-4-phosphate-5-kinase Iα (PIP5K Iα). DGKε is the only one of the 10 mammalian isoforms of DGK that exhibits arachidonoyl specificity and is the only isoform with the aforementioned motif. Mutations of the essential residues in this motif result in loss of arachidonoyl specificity. Furthermore, DGKα can be converted to an enzyme having this motif by substituting only one residue. When DGKα was mutated so that it gained the motif, the enzyme also gained some specificity for arachidonoyl-containing diacylglycerol. This motif is also present in an isoform of phosphatidylinositol-4-phosphate-5-kinase that we demonstrated had arachidonoyl-specificity for its substrate. Single residue mutations within the identified motif of this isoform result in loss of activity against an arachidonoyl substrate. The importance of acyl chain specificity for the phosphatidic acid activation of phosphatidylinositol-4-phosphate-5-kinase is also shown. We also demonstrate that the acyl chain dependence of this phosphatidic acid activation is dependent on the substrate. This is the first demonstration of a motif that endows specificity for an acyl chain in two studied enzymes, DGKε and PIP5K Iα.

Keywords

diacylglycerol kinase; acyl chain specificity; phosphatidylinositol-4-phosphate-5-kinase; arachidonic acid; PI-cycling

INTRODUCTION

Polyunsaturated fatty acids (PUFA) are essential nutrients for humans and are major components of cell membrane phospholipids. PUFA from the diet play an important role in the regulation of prostaglandin and proinflammatory cytokine synthesis.¹

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Address correspondence to: Richard M. Epand, Department of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, CANADA; Tel. 905 525-9140; Fax 905 521-1397; epand@mcmaster.ca.

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One of the most abundant PUFAs in mammalian cells is arachidonic acid (AA), and its derivatives are key mediators of a wide variety of physiological and pathophysiological processes, such as atherosclerosis,² arthritis, asthma and tumorigenesis.^{3, 4} AA is the precursor of a large family of bioactive compounds called eicosanoids, produced by cyclooxygenases and lipoxygenases.^{5, 6} Because of the potent biological actions of eicosanoids and of free AA itself, this fatty acid is maintained at very low levels in the cells, by being converted into cellular lipids by the enzymes arachidonoyl-CoA synthetase and lysophospholipid acyltransferases.⁷ Therefore, under physiological conditions, AA is generally found esterified at the *sn*-2 position of glycerophospholipids, such as choline and ethanolamine glycerophospholipids, phosphatidic acid and phosphatidylinositol.

To utilize the arachidonic acid pathway, the enzymes are required to distinguish the acyl chain length and saturation of the substrate. It is common that only one of the isoforms of a particular enzyme has specificity towards substrates with an arachidonate moiety. Thus, only the epsilon isoform of diacylglycerol kinase (DGK ϵ) shows substrate specificity *in vitro* for diacylglycerols with an arachidonoyl acyl chain at the *sn*-2 position.^{8–10} Phosphorylation of 1-stearoyl-2-arachidonoyl-glycerol (SAG) (for all lipid abbreviations, used in this study, refer to Table 1) is the first step in the resynthesis of phosphatidylinositols (PIs), and therefore, DGK ϵ contributes to the enrichment of 1-stearoyl-2-arachidonoyl species of PIs.^{11, 12} It is intriguing, that the domain responsible for the substrate recognition and specificity of DGK ϵ and other enzymes with arachidonate specificity has still not been identified. In the present study we propose a region located in the accessory domain of DGK ϵ that can recognize an arachidonoyl group. We identified a motif L-X_(3–4)-R-X₍₂₎-L-X₍₄₎-G, in which -X_(n)- represents n residues of any amino acids in this domain, that is present in DGK ϵ , as well as in phosphatidylinositol-4-phosphate-5-kinase type I. This motif is similar to a PUFA-recognizing domain recently identified in lipoxygenases (LOX) on the basis of a 1.85 Å resolution structure of an 8R-lipoxygenase from *Plexaura homomalla*, which reveals a U-shaped channel, defined by invariant amino acids that would allow substrate access to the catalytic iron.¹³ We showed that several residues in this motif are involved in the substrate specificity of two enzymes, acting on PUFA-containing substrates, DGK ϵ and PIP5K I α .

RESULTS

Mutations in the LOX-like motif of DGK ϵ greatly affect the activity of the enzyme

We identified a motif L-X_(3–4)-R-X₍₂₎-L-X₍₄₎-G in the accessory domain of DGK ϵ (Table 2), similar to that in lipoxygenases, but different in having an L, rather than an I as the first residue (Table 3). This motif is highly conserved in DGK ϵ among different species. To determine if the identified motif plays a role in the DGK ϵ specificity towards arachidonate-containing substrates, we mutated the residues in this region of the protein and measured the activities of FLAG-DGK ϵ WT and mutant proteins using the micelle-based assay with SAG as a substrate. The results showed that the mutations in this LOX-like region of DGK ϵ greatly affect the activity of the enzyme (Fig. 1). Notably, this effect is strongly correlated with the size and/or shape of a side chain of the mutated amino acids. From these results at a single substrate concentration, it is clear that all of the mutations result in a significant loss of enzymatic activity, even substitution of a single amino acid with a similar residue, thus demonstrating the importance of this region of DGK ϵ for enzymatic activity.

Affecting the substrate binding site would be expected to lower the activity of the enzyme against all substrates. However, if the binding site is specific for arachidonoyl groups, then the loss of activity should be greater for an arachidonoyl-containing substrate. In order to test this question more critically, we performed a kinetic analysis and calculated the Michaelis-Menten parameters for FLAG-DGK ϵ WT and its single residue mutants, L431I

and L438I, using SAG and SLG as substrates (Table 4). The arachidonoyl-containing substrate, SAG, is the major species of diacylglycerol in the PI-cycle. It was compared with a structurally similar diacylglycerol not containing arachidonic acid, SLG, as a critical test of the extent of arachidonoyl-specificity. Other more structurally different diacylglycerols, such as dioleoylglycerol or dipalmitoylglycerol, have very low activity with wild type DGK ϵ ^{12, 14} and also show very weak activity with mutant forms of this enzyme (data not shown). They therefore do not provide a critical comparison to test relative acyl chain specificity.

Almost all other mutants, L431S, L438S, L438A, L438G, and P439G, had very low activity (less than 2% of WT), which did not allow us to perform the kinetic analysis. Our results showed that k_{cat}/K_m was reduced for both L431I and L438I mutations of DGK ϵ . However the reduction of k_{cat}/K_m was much greater for SAG as substrate (four-fold decrease) compared with SLG (18% decrease). The number of substrate molecules turned over per enzyme molecule per second (k_{cat}), was also reduced, particularly with the substrate SAG. There was less affect on the affinity of the enzyme for these substrates as measured by K_m .

To test if the L431I and L438I mutations of FLAG-DGK ϵ affect the mode of inhibition by PA, we compared the inhibition of FLAG-DGK ϵ WT and mutant proteins by SOPA and SAPA (Fig. 2). Our results showed that L431I and L438I mutations of DGK ϵ do not affect the extent of PA inhibition.

Arachidonate preference can be introduced into DGK α by V656L mutation

We found that only one other isoform of DGK, DGK α , has a motif with some similarity to the LOX-like motif in DGK ϵ , but with a V656 residue instead of the Leu in DGK ϵ (Table 5). These motifs are found in the accessory domain of both DGK α as well as DGK ϵ . It was shown that DGK α does not have a preference for DAG with an arachidonate moiety.¹⁵ Therefore, to test if the arachidonate specificity could be introduced, at least to some extent, into DGK α , we mutated the V656 residue in human DGK α to L to obtain a protein with the same motif as in DGK ϵ (LxxxRxxLxxxxG) and compared the Michaelis-Menten parameters for 3 $\times$ HA-DGK α WT and the V656L mutant (Table 6). Our results showed that there is no statistically significant difference in the K_m or in the overall efficiency, V_{max}/K_m , of DGK α WT and V656L mutant, although it should be noted that there is an intrinsically large error in the determination of K_m . However, there is a difference in the V_{max} for these proteins. There was a significant 22% increase in V_{max} for SAG with the introduction of the mutation in contrast to a 16% decrease for SLG. When taken as a ratio of V_{max} for SAG to V_{max} for SLG, it shows a significant difference between DGK α WT and the V656L mutant proteins (Fig. 3).

PIP5K type I exhibits preference for arachidonoyl-PI(4)P

We found that phosphatidylinositol 4-phosphate 5-kinases (PIP5K) type I, but not type II, have a motif LxxxxRxxLxxxxG. There are three active isoforms of PIP5K type I, namely α , β and γ that are encoded by distinct genes. All three isoforms of the type I PIP5K have the same L-X₍₃₋₄₎-R-X₍₂₎-L-X₍₄₎-G motif as DGK ϵ mentioned above. In the present work we have used human PIP5K I α which has been suggested to fulfill a “housekeeping” function.¹⁶ To test if PIP5K type I forms have specificity towards an arachidonate -containing substrate, we compared the activity of human PIP5K type I α with SA-PI(4)P vs. DP-PI(4)P as substrates. There are few molecular forms of the substrate PI(4)P that have been synthesized. These two were chosen because of their availability. Our results showed that PIP5K type I α phosphorylates SA-PI(4)P about 8 times faster than DP-PI(4)P (Fig. 4). This is the first demonstration that this enzyme exhibits substrate acyl chain preference, although by itself it is not a critical test of arachidonoyl specificity.

To test the role of the identified motif in the activity of PIP5K type I α , we made L202I and L210I mutations in this region of the protein and performed a kinetic analysis of these mutant proteins in comparison with the WT PIP5K type I α . Our results showed that both L202I and L210I mutations decrease the substrate affinity and the enzyme efficiency of PIP5K type I α for arachidonate-containing PI(4)P (Table 7).

We also determined the activation of PIP5K I α with different PAs for both SA-PI(4)P (Fig. 5A) and DP-PI(4)P substrates (Fig. 5B). Our results using SA-PI(4)P as substrate showed that PIP5K is activated most by polyunsaturated PAs with the same fatty acids at *sn*-1 and *sn*-2 positions (di-PUFA-PA), such as DAPA, DLPA and DDPA. For the DP-PI(4)P substrate, PIP5K I α is activated 12 -fold by DAPA (Fig. 5B), showing that PIP5K can exhibit a very specific arachidonate preference for PA activation when DP-PI(4)P is used as substrate.

Discussion

Acyl chain specificity of DGK ϵ

We previously showed¹² that the inhibition and substrate specificity of DGK ϵ 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.¹² The inhibition of DGK ϵ by PA is competitive. Thus, the active site of DGK ϵ not only recognizes the lipid headgroup but also a combination of the two acyl chains in PA or DAG. Taken together, these findings suggest that the substrate-binding pocket of DGK ϵ should have a specific size and length, which is best suited for SAG. This isoform of DGK also contains a motif L-X₍₃₋₄₎-R-X₍₂₎-L-X₍₄₎-G. Mutations of several residues in this motif in DGK ϵ result in a marked loss of activity (Fig. 1). It should be pointed out that this marked sensitivity of the enzymatic activity of DGK ϵ to these single residue substitutions contrasts sharply with the very small changes in the kinetics of this enzyme that were observed when 58 residues were removed from the amino terminus of the enzyme.¹² In general, the less bulky the amino acid side chain in the mutated forms of the LOX-like motif, the lower the enzymatic activity.

The greater loss of activity by introducing mutations with less bulky amino acid side chains can be explained by the location of residues L438 and L431 at the bottom of the substrate - binding pocket, in analogy with the LOX enzyme,¹³ although the shape of this binding site may be different in DGK ϵ . In the case of DGK ϵ , the mutation of L438 or L431 residues to another amino acid with a smaller side chain would increase the volume of the substrate-binding pocket. The less fixed position of the substrate would then decrease the interaction between the hydroxyl group of the substrate and the phosphate group of the ATP that is bound to the ATP-binding site of the catalytic domain, which is outside of the acyl chain binding pocket. This would slow the rate of catalysis and the effect would depend on the size of the side chain of the mutated amino acid.

We also compared the mode of inhibition of FLAG-DGK ϵ WT and mutants L438I and L431I by PA (Fig. 2). These mutations have greatly affected k_{cat}/K_m (about 4 -fold decrease) for SAG, but not for SLG (Fig. 6). In contrast with this large decrease in the rate of phosphorylation of arachidonoyl substrates by mutations of the LOX-like motif, relatively little change in acyl chain specificity of PA inhibition is exhibited in these mutant forms of DGK ϵ compared with the wild type protein (Fig. 2). This can be explained by the fact that PA competes with the substrate for the substrate-binding site, but in the PA the hydroxyl group is already phosphorylated, and therefore, the distance of the PA from the ATP-binding site would not affect the extent of inhibition.

Furthermore, DGK shares the catalytic domain with other lipid kinases, such as sphingosine kinase and ceramide kinase,^{17, 18} but at the same time it is highly specific for DAG as substrate and does not catalyze the phosphorylation of sphingosine or ceramide. This suggests that the accessory domain is responsible for substrate recognition.¹⁹ The LOX-like motif, which we identified in DGK ϵ , is located in the accessory domain of DGK ϵ , further suggesting that it is involved in the substrate recognition and binding.

Acyl chain specificity of DGK α

Notably, DGK ϵ is the only DGK isoform that has an identified LOX-like motif, and it is the only isoform that has specificity for substrates with an arachidonate moiety. Although, one other isoform, DGK α , has a region with some similarity to that in DGK ϵ , but with a V656 residue instead of the Leu in DGK ϵ , and with the first Leu649 not being conserved among the mammalian species (Table 5).

Our results showed that the V656L mutation of DGK α that introduces a LOX-like motif in this isoform results in SAG having a higher $V_{\max}$ than SLG (Fig. 3). Therefore, this mutation increases the substrate preference of DGK α towards arachidonoyl substrates, making its substrate preference more similar to the DGK ϵ isoform. This supports our findings that the essential residues in the LOX-like motif play an important role in arachidonate-containing substrate specificity and recognition. The V656L mutant of DGK α is not as specific for arachidonoyl groups as is DGK ϵ , but perhaps one should not expect a newly introduced property in a protein by a single residue substitution to result in optimized function.

It is interesting, that DGK α also preferentially acts on substrates containing an arachidonoyl group when this group is incorporated into alkylacylglycerols.¹⁵ Although diacylglycerols are better substrates for DGK α than the alkylacylglycerols, no specificity is exhibited for arachidonoyl -containing diacylglycerols. This data may be explained by our observation that DGK α has a region similar to the LOX-like motif in DGK ϵ , but since it is not completely identical, DGK α does not exhibit specificity for arachidonoyl-containing DAG, unless this region is mutated so that the conserved residues are identical to those of DGK ϵ , as is shown in our study.

Acyl chain specificity of PIP5K I α

We identified a motif L-X₍₃₋₄₎-R-X₍₂₎-L-X₍₄₎-G in PIP5K type I, which converts PI(4)P to PI(4,5)P₂, and we confirmed that this enzyme exhibits preference for arachidonoyl-PI(4)P, and that the mutations in the identified region decrease the catalytic efficiency and substrate affinity for this enzyme (Table 7). Moreover, we also determined the activation of PIP5K I α with different PAs for both SA-PI(4)P and DP-PI(4)P substrates (Fig. 5). For DP-PI(4)P as substrate, the best activator of PIP5K I α is DAPA (Fig. 5B), showing that PIP5K exhibits arachidonate preference not only for the substrate, but also for its activator PA. The relationship between the arachidonoyl-requirement for the substrate of PIP5K I α and the acyl chain requirements for PA as an activator is not yet known. However, the role of PA for PIP5K as an activator clearly has to be different from the competitive inhibition that PA exhibits with DGK ϵ .¹² A previous study identified dilinoleoyl-PA as a potent activator of the phosphorylation of SA-PI(4)P by this enzyme.²⁰ Our results extend these findings and demonstrate that PIP5K stimulation by PA is sensitive to acyl chain composition and it depends on the substrate as well. We show that the PI-cycle intermediate, SAPA, is not the best activator for the phosphorylation of SA-PI(4)P, but rather it is di-PUFA-PA. The levels of di-PUFA-PA would be low in most tissues, but high in brain, where it would stimulate PI-cycling.

Conclusions

Enzymes and other proteins that interact with lipid exhibit specificity for certain lipid structures. In some cases, this is primarily a consequence of interacting with a lipid headgroup. An example is the PH domain that interacts with phosphatidylinositol phosphates with little specificity for the acyl chain. However, other proteins and enzymes can be specific for the nature of the acyl chain. Acyl chain specificity can have important physiological consequences, such as the segregation of arachidonoyl groups for signal transduction and perhaps other functions.

There has been current interest in the molecular basis of PUFA specificity. A recent proposal, based on the crystallographic structure of a coral lipoxygenase, has identified a pattern of amino acid residues that are important for recognizing arachidonic acid.¹³ In the present study we demonstrate that a similar amino acid pattern, L-X₍₃₋₄₎-R-X₍₂₎-L-X₍₄₎-G, is found in two other proteins that exhibit relative specificity for PUFA groups. While this motif does not predict a specific conformation of a PUFA binding site and this motif is found in other proteins that do not interact with PUFA groups, it has allowed us to identify the region in DGK ϵ that is involved in its unique arachidonoyl specificity among the 10 isoforms of mammalian DGK. We present further supporting evidence for this hypothesis by demonstrating the arachidonoyl specificity of DGK α can be increased by a site-specific mutation that results in the introduction of this proposed motif. In addition, we show that the enzyme PIP5K I α that also contains this motif exhibits specificity for PUFA moieties in both the substrate and in PA activators. Among the enzymes, lipoxygenases, DGK and PIP5K there are gross differences in the structure of the substrate and in the nature of the reaction that is catalyzed. One would therefore not expect *a priori* for the amino acid pattern or extent of acyl chain specificity to be identical in all three cases. One of the differences is that in lipoxygenases the first residue of this motif is an I, rather than L. Nevertheless, we demonstrate that there is a strong relationship between the amino acid pattern that we have identified and the property of PUFA specificity in two enzymes, DGK ϵ and PIP5K I α . It is also possible that a similar amino acid pattern can play a role in the substrate specificity and recognition of other enzymes with specificity towards PUFA-containing substrates, but further studies are needed to address this issue.

Amino acid patterns in forming structures that recognize particular features of substrates or ligands have been discovered in a number of proteins. There is also the so-called “CRAC” motif that has been proposed to be responsible for cholesterol recognition.^{21, 22} This amino acid pattern is also quite flexible in definition, it does not define a specific structure and the molecular basis of its relationship to cholesterol binding is not known. Nevertheless, there are an increasing number of examples of this motif being responsible for cholesterol interactions in proteins.²³⁻²⁷ Other examples include a phosphorylation site for Aurora B kinase, the mitosis-specific serine/threonine protein kinase, (R/K)1-3-X-(S/T) or (R/K)-(R/K)-X0-2-(S/T) where X denotes any amino acid;^{28, 29} the Phox homology domain for binding PI, (R/K)(R/K)(Y/F)xxFxxLxxxL or R(R/K)xxLxx(Y/F);³⁰ the lysosomal targeting sequences, Tyr -X-X-Hyd and LL (where Hyd is any hydrophobic amino acid).³¹ In all of these cases, as in the motif described in this paper, the motif is part of a structurally specific interaction site. However, the structure of this site is not determined solely by the motif with its large degree of variation and limited number of constraints. Nevertheless, identification of such motifs has been found to be a valuable tool in cell biology. Thus our manuscript extends this concept to PUFA recognition in two studied enzymes. This is of particular importance because of the roles of arachidonic acid in prostanoid metabolism and as an sn-2 acyl chain of lipids in the PI-cycle.

Materials and methods

DGK ϵ constructs

The FLAG epitope-tagged DGK ϵ , 3 $\times$ HA-DGK α and c-Myc-PIP5K type Ia expression vectors were prepared as previously described.^{32–34} All correspond to human forms of the respective enzymes. The mutants of FLAG-DGK ϵ , 3 $\times$ HA-DGK α and c-Myc-PIP5K type Ia were designed using the QuikChange Lightning Kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. The presence of the desired mutations was verified by sequencing analysis.

Cell culture

COS -7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO/Invitrogen) containing 10% fetal bovine serum (GIBCO/Invitrogen) at 37 °C in an atmosphere of 5% CO₂. The cells were grown to about 70%–80% confluency and transiently transfected with the expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 hours after transfection by scraping them into 1X PBS containing 1:100 protease inhibitor cocktail for use with mammalian cells and tissue (Sigma-Aldrich). The cells were pelleted at 5000g at 4 °C and kept at –90 °C until further use.

Immunoblot Analysis

Amounts of protein in the lysates of transfected COS -7 cells were quantified by immunoblotting. Protein samples for immunoblot analysis were prepared by incubation with 2% SDS buffer at 95 °C for 5 min. The resultant proteins were separated by 7.5% Tris-glycine SDS-PAGE and electroblotted onto an Immobilon-P polyvinylidenedifluoride membrane (Millipore). The membrane was then incubated with either a 1:2000 dilution of mouse anti-FLAGM2 (Sigma), 0.5 μ g/ml concentration of mouse THETM anti-HA tag IgG1 (GenScript, A01244), 1:800 dilution of mouse anti-c-Myc (Santa Cruz, sc-40), or 1:800 dilution of goat anti-actin (Santa Cruz, sc-1616) as the primary antibody and either a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse (Santa Cruz, sc-2005) or donkey anti-goat antibody (Santa Cruz, 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. A 3XFLAG-tagged bacterial alkaline phosphatase (3XFLAG-BAP) (Sigma-Aldrich) with a molecular mass of 49.9 kDa was used for DGK ϵ and its mutants as a standard in different lanes of the same blots.

Enzyme Preparations for Enzymatic Activity Assay

Prior to assay, cell pellets of COS-7 cells overexpressing human 3 $\times$ FLAG-DGK ϵ WT, 3 $\times$ HA-DGK α WT or mutants were resuspended in ice-cold cell 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 1:100 protease inhibitor cocktail for use with mammalian cells and tissue (Sigma-Aldrich)), allowed to lyse for 10 minutes on ice, sonicated for 5 minutes and then centrifuged at 100,000 g, 30 min at 4 °C. The supernatants were used in the assay of DGK activity. For PIP5K enzyme, cell pellets of COS-7 cells overexpressing human c-Myc-PIP5K type Ia were resuspended in ice-cold cell lysis buffer (2% (v/v) (octylphenoxy) polyethoxyethanol (Nonidet P-40), 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 10 μ g/mL aprotinin and leupeptin, 1 mM PMSF, 5 mM NaF, 100 μ g/mL soybean trypsin inhibitor, and 1:100 protease inhibitor cocktail for use with mammalian cells and tissue (Sigma-Aldrich)), allowed to lyse for 10 minutes on ice,

sonicated for 10 minutes and then incubated with agarose beads conjugated with anti-c-Myc antibodies (Santa Cruz, sc-40 AC) at 4 °C overnight. After that the beads were centrifuged and washed 1 time with IP kinase buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Triton X-100); 1 time with PBS pH 6.0, 0.5% Triton X-100; 1 time with 25 mM Tris, pH 8, 100 mM NaCl, 0.1% Triton X-100; 1 time with 25 mM Tris, pH 7.5, 500 mM NaCl, 0.1% Triton X-100; and 1 time with IP kinase buffer²⁰. After the final wash the beads were briefly centrifuged and resuspended in 1× assay buffer.

Quantification of Phosphatidic Acid and PI(4)P

The concentration of all PA and PI(4)P stocks used in this study was determined experimentally based on an assay for inorganic phosphate as described previously.³⁵ Briefly, 30 µl of 10% (w/v) Mg(NO₃)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 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 H₂SO₄ 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.

Detergent-Phospholipid-Mixed Micelle-based DGK Enzymatic Activity Assay

DGK was assayed for enzymatic activity using a detergent-phospholipid-mixed micelle-based protocol described by Walsh et al.⁹ as previously employed in our laboratory.³² 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 DGK ϵ) and/or 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS) (for DGK α)) were made at a constant total lipid concentration of 5.75 mM (see Table 1 for the list of the lipids). 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 MgCl₂, 4 mM EGTA, 1 mM dithiothreitol) containing 60 mM Triton X-100 and subsequently vortexing the hydrated lipid film for 2 min. Lysates from COS-7 cells expressing DGK were added to the mixed micelles along with double distilled H₂O to a final volume of 180 µL. The reaction was initiated by adding 20 µL of 1 mM [γ -³²P]-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 CHCl₃/CH₃OH, 0.25 mg/mL dihexadecyl phosphate). The organic layer was washed three times with 2 ml of wash solution (7:1 H₂O/CH₃OH, 1% HClO₄, 0.1% H₃PO₄). An aliquot of the organic layer was used to quantify the incorporation of ³²P 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. Negative controls were run with the addition of lysates from mock-transfected COS-7 cells and were confirmed to have activity levels significantly below lysates from cells overexpressing DGK. The measured activities of DGK enzymes could only be detected if exogenous lipid substrate was added, further indicating that endogenous lipids do not provide a sufficient concentration of substrate for their phosphorylation to be detected. In addition, the DGK activity of cells transfected with DGK ϵ is specific for SAG, while cells expressing DGK α can phosphorylate either SAG or SLG equally well. This finding is in accord with the known substrate specificities of these isoforms and is additional evidence that we are measuring the properties of the over-expressed enzyme. The activity measured with mock-transfected cells was subtracted from the values obtained using cells overexpressing one of the DGK ϵ or DGK α constructs. The assays were performed in triplicate and presented as the mean $\pm$ S.D. Each experiment was independently repeated at least two times. The day to day variations using the same enzyme preparation and the same lipids were not much greater than those for an individual experiment. The concentrations of

the individual lipid components of the mixed micelles are listed as their mole percentage of the detergent-phospholipid-mixed micelle, because DGKs are interfacial enzymes, and therefore, the concentrations of the individual lipid components at the surface of the mixed micelle are important in affecting DGK enzymatic activity rather than the bulk concentrations of the lipid components.

We also confirmed that the measured activity of DGK is not substantially affected by product degradation as a result of the hydrolysis of PA by endogenous PA phosphatases (PAP), or as a result of the upregulation by endogenous protein kinase C (PKC) and phospholipases D (PLD). Our results showed that the DGK ϵ activity measured in the absence and presence of either propranolol (PAP³⁶ and PKC³⁷ inhibitor, from Sigma-Aldrich), bromoenol lactone (BEL, PAP inhibitor,³⁸ from Sigma-Aldrich) (Fig. 7A), or PLD inhibitors (Avanti Polar Lipids, equimolar mix of VU0359595,³⁹ VU0155056⁴⁰ and VU0285655–1⁴¹ inhibitors) (Fig. 7B) are not significantly different.

Detergent-Phospholipid-Mixed Micelle-based PIP5K Enzymatic Activity Assay

PIP5 kinase activity assay was performed as described by Parker et al.⁴² with the following modifications. Reactions were performed in a 100 μ L reaction volume in a standard buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 1 mM EGTA, 0.1% Triton X-100 and 50 μ M [γ -³²P]ATP (2 μ Ci/reaction). The reaction was stopped after 10 min by the addition of 500 μ L of 1 N HCl and 2 mL of chloroform:methanol (1:1) simultaneously. The assay was washed twice with 1 mL of methanol:1N HCl. An aliquot of the organic layer was used to quantify the incorporation of ³²P into PI(4,5)P₂ using Cerenkov counting. Negative controls were run with the addition of beads immunoprecipitated from mock-transfected COS-7 cells and were confirmed to have activity levels significantly below immunoprecipitates from cells overexpressing PIP5K. Kinetic parameters were calculated using the effective concentration of PI4P at the surface of the micelle. To be consistent with previously presented data on this enzyme,⁴² the effective surface concentration of PI(4)P was calculated by multiplying the mole fraction of PI(4)P at the surface of the micelle by the total concentration of PI(4)P.

Kinetic Analysis of the Micelle-Based Assay of DGK and PIP5K Activity

The Michaelis-Menten constants, $V_{\max}$ and K_m , were evaluated by a nonlinear regression analysis (initial velocity (v_0) versus substrate concentration ([S])), as well as by using Hanes plots ([S]/ v_0 versus [S]). The content of DGK and PIP5K was determined using immunoblot analysis as described above. Microcal Origin software was used to determine k_{cat} , $V_{\max}$ and K_m parameters.

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Abbreviations

AA	arachidonic acid
BEL	PAP inhibitor
DAG	diacylglycerol
DGK	diacylglycerol kinase

LOX	lipoxygenase
PA	phosphatidic acid
PAP	PA phosphatases
PI	phosphatidylinositol
PI(4	5)P ₂ , phosphatidylinositol-4,5-bisphosphate
PI(4)P	phosphatidylinositol-4-phosphate
PIP5K	phosphatidylinositol 4-phosphate 5-kinase
PKC	protein kinase C
PLD	phospholipases D
di -PUFA-PA	phosphatidic acid with two polyunsaturated acyl chains
PUFA	polyunsaturated fatty acid; see also Table 1 for lipid nomenclature used.

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Figure 1.

A. Comparison of the enzyme activities for FLAG-DGKε WT and mutants L438M, L438I, L438S, L438A, L438G, L431I, L431S, P439G. Enzyme activity is expressed as a percentage of the activity of FLAG-DGKε WT protein with SAG as a substrate. B. Western blots showing the expression levels of DGKε WT and mutants (anti-FLAG panel), and the expression levels of actin (anti-actin panel) in the transfected cells. Enzyme activity was normalized for the amount of protein in the lysates. The activity measured with mock-transfected cells was normalized for the amount of actin and subtracted from the values obtained using cells overexpressing one of the DGKε constructs. The activity measured with mock-transfected cells was on average 5–10% of the activity measured with DGKε WT.

Figure 2.

Comparison of inhibition of DGK ϵ by PA. DGK enzymatic activity was measured with 15 mM Triton X-100, 0.1 mM [γ - 32 P]-ATP, and 2 mol % SAG (shown as dark grey bars) or with the addition of either 2 mol % SAPA (light grey bars) or SOPA (white bars). The numbers above the bars show the enzymatic activity of proteins in presence of PA as a percentage of the enzymatic activity in the absence of PA.

Figure 3.

Comparison of SAG to SLG ratios for $V_{\max}$ parameters for 3×HA-DGK α WT and V656L mutant proteins. V656L mutation of DGK α increases the arachidonoyl preference of the enzyme. *The difference between $V_{\max}$ ratios of SAG to SLG for DGK α WT and V656L mutant is statistically significant with $P < 0.05$.

Figure 4.

Comparison of the enzyme activities for c-myc-PIP5K I α with SA-PI(4)P and DP-PI(4)P as a substrate. As source of SA-PI(4)P, brain PI(4)P (Avanti Polar Lipids) was used. Only SA-PI(4)P and DP-PI(4)P were compared, since other PI(4)P are not commercially available. Negative control (EV) is performed with SA-PI(4)P as a substrate with the addition of beads immunoprecipitated from mock-transfected COS-7 cells.

Fig. 5. Activation of PIP5K by PA with A) SA-PI(4)P and B) DP-PI(4)P as substrate. As source of SA-PI(4)P, brain PI(4)P (Avanti Polar Lipids) was used. PIP5K enzymatic activity was measured with 20 μ M PI(4)P and 50 μ M PA.

Figure 6.

Graphic presentation of the kinetic parameters for FLAG-DGKε WT and its L438I and L431I mutants. Results are presented as the mean $\pm$ S.D. A. Results show that k_{cat}/K_m is greatly affected (about 4-fold decrease) by L438I and L431I mutations of DGKε for SAG, but not SLG substrate. B. Comparison of K_m parameters shows that L438I and L431I mutations of DGKε slightly affect the substrate affinity of the enzyme, decreasing the preference for SAG over SLG. C. An example of kinetic data of 3 independent experiments (Exp1, Exp2 and Exp3) is shown for FLAG-DGKε WT with SAG as a substrate. Every experiment is performed in triplicate. A nonlinear regression curve fitting Exp1 data is shown in red.

Figure 7.

Comparison of the FLAG-DGKε enzyme activities in the absence and presence of PA phosphatase (PAP) inhibitors, protein kinase C (PKC) and phospholipase D (PLD) inhibitors. A. Propranolol (PAP and PKC inhibitor) and bromoenol lactone (BEL, PAP inhibitor) do not affect the measured DGKε activity. B. 6 μM or 120 μM PLD inhibitors (Avanti Polar Lipids, equimolar mix of VU0359595, VU0155056 and VU0285655-1 inhibitors) do not significantly affect the measured DGKε activity. Negative control (EV) is performed with the lysates from COS-7 cells transfected with empty vector (p3XFLAG-CMV-7.1, Sigma-Aldrich).

Table 1

Lipids used and/or referred to in this study

Abbreviation	Full name	Alternative notation (<i>sn-1/sn-2</i>)
DAG		
AAG	1-Arachidoyl-2-arachidonoyl- <i>sn</i> -glycerol	20:0/20:4 DAG
DOG	1,2-Dioleoyl- <i>sn</i> -glycerol	18:1/18:1 DAG
PAG	1-Palmitoyl-2-arachidonoyl- <i>sn</i> -glycerol	16:0/20:4 DAG
SAG	1-Stearoyl-2-arachidonoyl- <i>sn</i> -glycerol	18:0/20:4 DAG
SLG	1-Stearoyl-2-linoleoyl- <i>sn</i> -glycerol	18:0/18:2 DAG
PA		
AAPA	1-Arachidoyl-2-arachidonoyl phosphatidic acid 1,2-	20:0/20:4 PA
DAPA	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- <i>sn</i> -glycero-3-phosphocholine	18:1/18:1 PC
PS		
DOPS	1,2-Dioleoyl- <i>sn</i> -glycero-3-phospho-L-serine	18:1/18:1 PS
PI4P		
SA-PI4P	1-Stearoyl-2-arachidonoyl phosphatidylinositol-4- phosphate	18:0/20:4 PI4P
DP-PI4P	1,2-Dipalmitoyl phosphatidylinositol-4-phosphate	16:0/16:0 PI4P

Table 2

A partial sequence alignment of vertebrate DGK ϵ . The conserved residues, similar to those in LOX, are colored in red. Note the high degree of sequence conservation in this region of the protein from avian to mammalian species.

NP_003638 [<i>Homo sapiens</i>]	421	KDLNKKVELELDGERVALPSLEGHIVLNIGYWG	453
NP_062378 [<i>Mus musculus</i>]	418	KDLNKKIELELDGERVELPNLEGHIVLNIGYWG	450
XP_618342 [<i>Bos taurus</i>]	418	KDLNKKVELELDGERVELPNLEGHIVLNIGYWG	450
XP_001503369 [<i>Equus caballus</i>]	418	KDLNKKVELELDGERVELPNLEGHIVLNIGYWG	450
XP_548222 [<i>Canis familiaris</i>]	418	KDLNKKIELELDGERVELPNLEGHIVLNIGYWG	450
XP_001521727 [<i>Ornithorhynchus anatinus</i>]	414	KDLNKKVELELDGERVELPNLEGHIVLNIGYWG	446
XP_523803 [<i>Pan troglodytes</i>]	421	KDLNKKVELELDGERVALPSLEGHIVLNIGYWG	453
XP_001234226 [<i>Gallus gallus</i>]	421	KDLNKKVELELDGERIELPNLEGHIVLNIGYWG	453
XP_002192399 [<i>Taeniopygia guttata</i>]	407	KDLNKKVELELDGERIELPNLEGHIVLNIGYWG	439

Table 3

A partial sequence alignment of LOX. The conserved residues, located in U-shaped channel, that would allow substrate access to the catalytic iron, ¹³ are colored in red.

LOX15 [<i>Oryctolagus cuniculus</i>]	389	LIVPHLRYTLEINVRARNGLVSDFGIFDQIM	419
LOX15 [<i>Homo sapiens</i>]	388	LIIPHLRYTLEINVRARTGLVSDMGIFDQIM	418
LOX12 [<i>Homo sapiens</i>]	388	FLIPHIRYTMEINTRARTQLISDGGIFDKAV	418
LOX12 [<i>Rattus norvegicus</i>]	389	LLVPHLLYTMEINVRARSDLISERGFFDKAM	419
LOX5 [<i>Rattus norvegicus</i>]	395	LLVAHVRFITIAINTKAREQLNCEYGLFDKAN	425
LOX1 [<i>Arabidopsis thaliana</i>]	547	LLEPHFRDTMNINALARQILINGGIFEITV	577

Table 4

Summary of the kinetic parameters for FLAG-DGK ϵ WT and its L438I and L431I mutants. Results are presented as the mean $\pm$ S.D. Although the measured activity at one substrate concentration did not show differences in substrate specificity (Fig.1), a more complete analysis of the kinetics does clearly illustrate that both k_{cat} , that represents the catalytic rate constant, as well as k_{cat}/K_m , that represents the pseudo first order rate constant at low substrate concentrations, are affected by the mutations, particularly for the SAG substrate (see Fig. 7 for the graphic representation of Table 4).

	K_m , mol%	k_{cat} , sec $^{-1}$	k_{cat}/K_m , sec $^{-1}$ mol% $^{-1}$
<u>FLAG-DGKϵ WT</u>			
SAG	2.44 $\pm$ 0.14	10.2 $\pm$ 0.3	4.16 $\pm$ 0.14
SLG	7.0 $\pm$ 1.4	4.6 $\pm$ 0.6	0.66 $\pm$ 0.11
<u>FLAG-DGKϵ L438I</u>			
SAG	1.88 $\pm$ 0.15	2.24 $\pm$ 0.07	1.19 $\pm$ 0.05
SLG	2.9 $\pm$ 0.5	1.56 $\pm$ 0.09	0.54 $\pm$ 0.04
<u>FLAG-DGKϵ L431I</u>			
SAG	2.4 $\pm$ 0.6	2.1 $\pm$ 0.2	0.91 $\pm$ 0.14
SLG	5.1 $\pm$ 1.2	2.7 $\pm$ 0.4	0.53 $\pm$ 0.09

Table 5

A partial sequence alignment of mammalian DGK α . The conserved residues, similar to those in LOX, are colored in red.

NP_963848 [<i>Homo sapiens</i>]	639	PDILKTCVPDLSDKRLEVVGLEGAIEMGQIYTK	671
NP_058091 [<i>Mus musculus</i>]	634	PDILKTCVPDMSDKRLEVVGIEGAIEMGQIYTR	666
NP_001071328 [<i>Bos taurus</i>]	638	PDILKTCVPDLSDKRLEVVGLEGAIEIGQIYTK	670
NP_542965 [<i>Rattus Norvegicus</i>]	631	PDILKTCVPDMSDKRLEVVGIEGVIEMGQIYTR	663
XP_855720 [<i>Canis familiaris</i>]	477	PDILKTCVPDLTDKRLEVVGLEGAIEMGQIYTK	509
XP_001112067 [<i>Macaca mulatta</i>]	527	PDILKTCVPDLSDKRLEVVGLEGAIEMGQIYTK	559
NP_999197 [<i>Sus scrofa</i>]	638	PDILKTCVPDLSDKRLEVVGLEGAIEMGQIYTK	670
XP_001169813 [<i>Pan troglodytes</i>]	527	PDILKTCVPDLSDKRLEVVGLEGAIEMGQIYTK	559

Table 6

Summary of the kinetic parameters for 3×HA-DGKα WT and V656L mutant. Kinetic analysis shows that the V656L mutation of DGKα increases the $V_{\max}$ of the enzyme for SAG and decreases it for SLG, thus introducing the arachidonoyl preference (see also Fig. 3B). Values of $V_{\max}$ are relative values since the absolute amount of enzyme in the cell preparations is not known. $V_{\max}$ of 3×HA-DGKα V656L is normalized for the amount of protein relatively to WT. Results are presented as the mean $\pm$ S.D.

	K_m , mol%	$V_{\max}$, nmol PA min ⁻¹	$V_{\max}/K_m$, mol% ⁻¹ sec ⁻¹
3×HA-DGKα WT			
SAG	2.1 $\pm$ 0.4	1.44 $\pm$ 0.09	0.70 $\pm$ 0.13
SLG	2.8 $\pm$ 0.3	1.51 $\pm$ 0.08	0.55 $\pm$ 0.07
3×HA-DGKα V656L			
SAG	1.8 $\pm$ 0.3	1.75 $\pm$ 0.11	1.00 $\pm$ 0.17
SLG	1.5 $\pm$ 0.4	1.26 $\pm$ 0.12	0.87 $\pm$ 0.25

Table 7

Summary of the kinetic parameters for c-myc-PIP5K I α WT and L202I and L210I mutants. Our results show that L202I and L210I mutations of PIP5K I α significantly decrease the substrate affinity of the enzyme (12-fold increase in K_m for L202I mutant and 2-fold for L210I). Consequently, the mutations also affect the enzyme efficiency (about 6-fold decrease in V_{max}/K_m for L202I and 2-fold for L210I). Values of V_{max} are relative values since the absolute amount of enzyme in the cell preparations is not known. V_{max} for PIP5K I α L202I and L210I mutants is normalized for the amount of proteins relatively to WT. Kinetic parameters are calculated using the effective concentration of PI4P at the surface of the micelle. The effective surface concentration of PI(4)P was determined by multiplying the mole fraction of PI(4)P at the surface of the micelle by the total concentration of PI(4)P to be comparable to previously published work.⁴⁵ Results are presented as the mean $\pm$ S.D.

K_m , μ M	V_{max} , nmol/min	V_{max}/K_m , 1/ μ M/min
<u>PIP5K Iα WT</u>		
0.3 ± 0.1	0.125 ± 0.008	0.35 ± 0.10
<u>PIP5K Iα L202I</u>		
3.6 ± 0.7	0.200 ± 0.027	0.06 ± 0.01
<u>PIP5K Iα L210I</u>		
0.7 ± 0.2	0.097 ± 0.012	0.14 ± 0.05