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Dramatic Differences in the Roles in Lipid Metabolism Of Two Isoforms of Diacylglycerol Kinase[†]

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Abstract

Lipid species change for SV40 transformed fibroblasts from wild-type or from diacylglycerol kinase- ϵ (DGK ϵ) or diacylglycerol kinase- α (DGK α) knock out mice, were determined for glycerophospholipids, poly-phosphatidylinositides (GPIsP_n), and diacylglycerol (DAG) using direct infusion mass spectrometry. Dramatic differences in arachidonate (20:4 fatty acid)-containing lipids were observed for multiple classes of glycerophospholipids and polyphosphatidylinositides between wild-type and DGK ϵ knock out cells. However, no difference was observed in either the amount or the acyl chain composition of DAG between DGK ϵ knock out and wild-type cells, suggesting that DGK ϵ catalyzed the phosphorylation of a minor fraction of the DAG in these cells. The differences in arachidonate content between the two cell lines were greatest for the GPIsP_n lipids and least for DAG. These findings indicate that DGK ϵ plays a significant role in determining the enrichment of GPIsP_n with 20:4 and that there is a pathway for the selective translocation of arachidonoyl-phosphatidic acid from the plasma membrane to the endoplasmic reticulum. In contrast, no substantial difference was observed in the acyl chain composition of any class of glycerophospholipid or diacylglycerol between lipid extracts from fibroblasts from wild-type mice or from DGK α knock out mice. However, the cells from the DGK α knock out mice had a higher concentration of DAG, consistent with the lack of down regulation of the major fraction of DAG by DGK α , in contrast with DGK ϵ that is primarily responsible for enrichment of GPIsP_n with arachidonoyl acyl chains.

Eukaryotic diacylglycerol kinases (DGKs)¹ are a family of 10 known isoforms playing important roles in signal transduction (1–3). In this work we compare the properties of two isoforms of DGK, DGK α and DGK ϵ . Although these two isoforms catalyze the same reaction,

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¹Abbreviations: DGK, diacylglycerol kinase; RT, reverse transcriptase; DAG, diacylglycerol; GPA, phosphatidic acid; GPEtn, phosphatidylethanolamine; GPCho, phosphatidylcholine; GPIs, phosphatidylinositol; GPGro, phosphatidylglycerol; GPSer, phosphatidylserine; PLC, phospholipase C; GPIs(4,5)P₂, phosphatidylinositol (4,5) bisphosphate; WT, wild type; KO, DGK ϵ -knockout or DGK α -knockout; GPIsP_n, polyphosphatidylinositides; ESI/LC/MS/MS, electrospray ionization/liquid chromatography/tandem mass spectrometry.

they have very different modes of interaction with membranes and different mechanism of regulation. DGK α contains two E-F hands and is a Ca²⁺-dependent enzyme (4,5), although it can also exhibit Ca²⁺-independent activity when binding to membranes rich in phosphatidylethanolamine (GPEtn) or cholesterol (6). DGK α is an amphitropic enzyme that binds to membranes only upon activation (7,8). In contrast DGK ϵ is the only isoform with a hydrophobic segment that promotes attachment of the protein to membranes (9). In addition, DGK ϵ is also unique in being the only isoform of DGK that has specificity for DAG containing arachidonoyl chains (10,11) as well as other polyunsaturated acyl chains (12). Hence, the DAG derived from GPIs(4,5)P₂ hydrolysis by GPIs(4,5)P₂-specific phospholipase C (PLC) will also be arachidonoyl-rich as will the phosphatidic acid (GPA) formed as a product of DGK ϵ -catalyzed phosphorylation. This process is cyclical and the DAG that is phosphorylated by DGK to produce GPA, in turn can be converted to GPIs(4,5)P₂ by reaction with CDP-inositol. We studied the contribution of DGK ϵ in determining the enrichment of GPIs(4,5)P₂ with arachidonic acid.

Studies using DGK ϵ -deficient mice have demonstrated a role for this isoform in regulating seizure susceptibility and long-term potentiation (13,14). It has also been shown that upon electroconvulsive shock there was a reduced accumulation of arachidonoyl-DAG as well as free arachidonic acid (14). There is also a smaller reduction in the level of GPIs(4,5)P₂ in the knock out mice upon electroconvulsive shock (14). In the present work we study the role of DGK ϵ in the distribution of arachidonoyl side chains among different lipids in the basal state without stimulation. Another isoform, DGK α , is a negative regulator of antigen-mediated triggering of the T-cell receptor (15). This isoform has chemotactic, proliferative and angiogenic activity (16). Studies with DGK α knock out mice demonstrated an impairment of anergy induction (17). T-cells from these knock out mice produce more interleukin-2 and show increased proliferation in response to T-cell receptor activation (17). DGK α is expressed in several human melanoma cell lines but not in noncancerous melanocytes (18). It has also recently been found that this isoform can play an important role in the metastasis of certain breast cancers (19). Hence DGK α and DGK ϵ appear to have different biological functions. We have undertaken a lipidomics study to determine if there are also differences in lipid processing resulting from the deletion of each of these two DGK isoforms.

Experimental Procedures

Tissue culture

Mouse fibroblasts were obtained from embryos of mice that were made deficient in DGK ϵ ($-/-$) (14) or in DGK α ($-/-$) (17). In each experiment these cells were compared with wild-type embryonic fibroblasts obtained from siblings of the ($-/-$) mice. These cells derived from DGK ϵ ($+/+$) or DGK α ($+/+$) embryos, respectively, are designated WT. All cells were immortalized by transfection with the SV40 large T antigen. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 25 mM HEPES, and 1% Penicillin/Streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Expression levels of DGK in cell lines

We compared the level of mRNA of DGK ϵ or DGK α from the mouse embryonic fibroblast cell lines described above using reverse transcriptase (RT) PCR. These levels were compared with those of actin mRNA. Briefly, total RNA was isolated from each of the four cell lines and 1 μ g of RNA was reverse transcribed using Moloney-Murine Leukemia Virus (MMLV) reverse transcriptase. Using 3% of the cDNA, DGK α , DGK ϵ , and actin were amplified for 36 cycles by annealing at 64 °C for 1 min and extending at 72 °C for 1 min. The primers used for DGK α were 5'-ATGGCCAAAGAGAAGGGCCTC + 5'-GCAGTGGCACTGTGGTAGCCCC and those for DGK ϵ were 5'-CTCAGTTCGAGTCCTTGTTG + 5'-

ATAGACGCCAACGATTTCCAG. Equal aliquot of the resulting reaction was subjected to agarose gel electrophoresis.

Glycerophospholipids analysis

Global lipid extracts were prepared via a modified Bligh/Dyer extraction procedure as described previously (20). Briefly, cells were scraped in 800 μ L of ice-cold 0.1N HCl/methanol (1:1) and 400 μ L of CHCl_3 was added to the suspension. The samples were vortexed for 1 min, layers separated by centrifugation at 18000g for 5 min at 4°C and 20 μ L of an internal standard containing equimolar amounts of 22:0 GPCho (1,2-diundecanoyl-*sn*-glycero-3-phosphocholine) and 28:0 GPA (1,2-dimyristoyl-*sn*-glycero-3-phosphate) were added to the organic layer before drying *in vacuo*. Dried lipids were redissolved in 80 μ L of $\text{CH}_3\text{OH}:\text{CHCl}_3$ (9:1). Mass spectral analysis was performed on a Finnigan TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a Harvard Apparatus syringe pump and electrospray source. Samples were analyzed at an infusion rate of 10 μ L/min in both positive and negative ionization modes over the range of m/z 350–1200. Data were collected with the Excalibur software package (ThermoFinnigan) and analyzed with software developed in our laboratory as described in (21). Identification of individual glycerophospholipids was accomplished by tandem mass spectrometry (ESI/LC/MS/MS). Extracted lipids were dissolved in 100 μ L of isopropanol (IPA):hexane:100 mM $\text{HCOONH}_{4(\text{aq})}$ 58:40:2 (mobile phase A). We utilized an Applied Biosystems/ MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/ linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA). Coupled to this instrument were a Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) HPLC system consisting of a SCL 10 AVP controller, two LC 10 ADVP pumps and a CTC HTC PAL autosampler (Leap Technologies, Carrboro, NC). All samples were separated on a Phenomenex (Phenomenex, Torrance, CA) Luna Silica column (2 $\times$ 250 mm, 5 μ particle size) using a 20 μ L sample injection. Lipids were separated using a binary gradient program consisting of IPA: hexane: 100 mM $\text{HCOONH}_{4(\text{aq})}$ 58:40:2 (mobile phase A), and IPA:hexane:100 mM $\text{HCOONH}_{4(\text{aq})}$ 50:40:10 (mobile phase B). The following LC gradient was used: 0–5min, B=50%; 5–30min, B=50%-100%; 30–40min, B=100%; 40–41min, B=100%-50%; 41–50min, B=50%, delivered at a flow rate of 0.3ml/min. This analysis results in class separation and fragmentation of the individual species within the class thus allowing for a more precise identification (20,22).

Phosphatidylinositol phosphate (GPI_{ns}P_n) Determination

Polyphosphatidylinositides extraction was performed as described previously (23), with the following modifications: dried lipids were rapidly redissolved in 55 μ L of 1:1:0.3 $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$. Before analysis, 5 μ L of 300 mM piperidine_(aq) and 1 μ L of (0.11 M) internal standard 1,2-diocanoyl-*sn*-glycero-3-phosphoinositol-4,5-bisphosphate was added to each sample, and the sample was vortexed and centrifuged briefly prior to mass spectral analysis by direct infusion on a MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA).

DAG analysis

Analysis and quantification of DAG species from cell extracts was performed according to the procedure described in (24). After phospholipids extraction by the modified Bligh/Dyer procedure diacylglycerols were isolated by separation using normal phase column chromatography and isocratic elution with 65:35:0.7 $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$. 1,2-dilauroyl-*sn*-glycerol (100 ng) was used as an internal standard and added to each sample before column separation.

Results

RT-PCR of DGK

The results of the RT-PCR analysis confirm that the wild type (WT) cells express both DGK α as well as DGK ϵ . However, no mRNA for DGK α or for DGK ϵ was detected in the respective knock out (KO) cell lines (Fig. 1). As a positive control we show that mRNA for actin is detected in comparable amounts in the KO and WT cell lines.

Phospholipid composition of wild type (WT) versus DGK ϵ knockout (KO) cells

Glycerophospholipid profiles for six major classes show significant differences in arachidonate containing species between the WT and KO cells. The arachidonate-containing lipids are compared to all others that do not possess this acyl substituent between WT and KO cells (Fig. 2). The lipid class showing the greatest difference in arachidonoyl content between WT and KO cells is polyphosphoinositides (GPIsP_n), which is the sum of all phosphatidylinositols including GPIs, GPIsP, GPIs(4,5)P₂ and GPIs(3,4,5)P₃. More specifically, the deletion of DGK ϵ decreases the 38:4 GPIs content from approximately 33% to 24% of the GPIs lipid pool. To compensate for this, it appears that the KO cells increase the incorporation of oleate (18:1 fatty acid) into the inositol lipid fraction (Fig. 3). The identity of each glycerophospholipid component was resolved by tandem mass spectrometry and their fatty acyl composition was determined. These data are compiled in the supplementary Tables available at <http://pubs.acs.org>. In the case of 36:2 GPIs, for example, 18:1/18:1 fatty acids are the predominant species. The 36:2 GPIs seems to have a significantly higher content in the KO fibroblasts with an increase from around 6 to 11% of the class. Within the GPIsP_n lipids, a separate analysis showed that there was a marked change in arachidonate- and oleate-containing lipids following knockout of DGK ϵ for GPIs, GPIsP, GPIs(4,5)P₂ (Fig. 4). Due to the low abundance of basal GPIs(3,4,5)P₃, neither 36:2 nor 38:4 GPIs(3,4,5)P₃ were detectable in these experiments.

The glycerophosphatidic acid (GPA) lipids had a similar pattern to that observed in the GPIs lipid pool, although the differences between KO and WT were somewhat reduced. Again in this class, 38:4 GPA was the most affected by the deletion of DGK ϵ , with the KO cells having reduced levels 38:4 GPA. To balance this loss, the KO cells have increased amounts of saturated and monounsaturated fatty acid containing species such as 36:2 GPA.

Glycerophosphatidylethanolamine (GPEtn) lipids have a more complex lipid pattern due to the presence of plasmalogen and plasmalogen (ether and vinyl ether) containing lipids in addition to the usual diacyl lipids. Numerous arachidonate containing GPEtn lipids (38:4, 38:5, 38:6, as well as the 38:4 and 38:5 plasmalogen/plasmalogen GPEtn for example) all contribute less to the KO fibroblast GPEtn lipid pool compared to the WT cells. However, overall the difference in arachidonoyl-containing GPEtn between KO and WT cells is much less than the differences observed for GPIs. Glycerophosphatidylcholines (GPCho) also had easily observable differences in arachidonate-containing lipid profiles following DGK ϵ knockout. The 38:4 GPCho contributes more to the total GPCho lipid pool in the WT cells compared to the KO cell type. To compensate for the loss of 38:4 GPCho, it appears that the KO cells had a higher fraction of saturated and monounsaturated fatty acid containing GPCho (Fig. 5). Thus, in summary, most lipid classes show greater 38:4 than 36:2 lipids in the WT compared with KO cells, indicating a loss of arachidonoyl-containing lipids.

We have extensively characterized the glycerophospholipid species from both WT and DGK ϵ -KO cell extracts by ESI/LC/MS/MS (Supplemental Table 1&Supplemental Table 2). As a result of this analysis 366 species were identified in WT and 405 species in DGK ϵ -KO cell extracts. Only two of the phospholipid classes show marked differences in terms of the number and variety of the identified species. More phosphatidylserine (GPSer) (50 species vs.

33) and a rare phospholipid, phosphatidylthreonine (GPThr) (25), (20 species vs. 7) species were ascertained in the DGK ϵ -KO cell extracts.

We also determined the ratio of GPA to GPIs for both WT and DGK ϵ -KO cells (Fig. 6). This ratio was significantly higher for the DGK ϵ -KO cells ($p=0.0014$ by ANOVA with random effects). The analysis of DAG species from both genotypes reveals no significant differences. The lack of measured changes in DAG might have resulted because there were only trace levels of 38:4 DAG in both WT and KO cell lines. The majority of the lipids present were from the 32, 34, and 36 series of DAGs.

Phospholipid composition of wild type (WT) versus DGK α knockout (KO) cells

Analysis of the glycerophospholipids from WT and DGK α KO cells was also performed. The cellular phospholipid pattern did not have broad, statistically significant differences in glycerophospholipid species between the WT and the KO. In GPIs only 36:4 does have a modest but significant decrease in class percentage (Fig. 7). The difference in the ratio of GPA to GPIs between the DGK α KO and WT cells was marginally significant ($p=0.0498$ by ANOVA with random effects). See Fig. 6.

Comprehensive ESI/LC/MS/MS analysis of the glycerophospholipid species in these two phenotypes did not reveal any significant differences. 407 phospholipid species were identified in WT and 348 species in DGK α KO cell extracts (Supplemental table 3&Supplemental table 4). In contrast, the analysis of DAG species revealed some interesting differences between WT and KO. Here too the majority of the DAG species were from the 32, 34 and 36 series but unlike in the DGK ϵ KO case, there were marked changes in the quantity of DAG species in the extracts from DGK α KO. The amount of DAGs is almost double in the KO samples compared to WT. The largest differences occur in saturated, mono- and di- unsaturated DAG species (Fig. 8).

Discussion

The two isoforms of DGK that we studied in this work are known to have different biological functions. One reason for this is that these enzymes are more highly expressed in different organs. DGK ϵ is found principally in brain, retina and cardiac muscle, while DGK α is found mainly in white blood cells, spleen and thymus. Both DGK α and DGK ϵ are expressed in the WT cell lines that we used, but they are not expressed in the corresponding KO cell line (Fig. 1). The present work demonstrates that these two isoforms of DGK also have very different properties on a biochemical level. We suggest that DGK ϵ is involved more specifically in GPIsP_n cycling, while DGK α contributes to regulating the bulk concentration of DAG in the cell from all sources.

DGK ϵ contributes significantly to the arachidonoyl enrichment of GPIs(4,5)P₂ since the KO cells have less arachidonoyl-GPIs(4,5)P₂ (Fig. 3 and Fig. 4). This is the case in spite of the fact that the specificity of DGK ϵ for arachidonoyl-containing forms of DAG is only about 10-fold using *in vitro* assays (9). Even more surprising is the increase in arachidonoyl-enrichment between the formation of GPA by DGK ϵ and its conversion to GPIs(4,5)P_n (Table 1). In the present work we observed that the arachidonoyl content of DAG is only about 3% and there is little difference between KO and WT cells. This increases to slightly over 10% arachidonoyl content of GPA. The increase is less for KO cells than for WT, indicating that DGK ϵ contributes to the arachidonoyl-enrichment of GPA as would be expected because of the arachidonoyl-specificity for substrates phosphorylated by this DGK isoform. There are also other sources of GPA, in addition to DGK ϵ , including other DGK isoforms as well as from phospholipase D-catalyzed reactions. In the case of phospholipase D, substrates with an arachidonoyl group on

the *sn*-2 of the glycerol backbone are not favored by the enzyme (26), indicating that this is not a source of arachidonoyl enrichment.

The GPA that is formed can be converted back into GPIs(4,5)P₂. The first step in this conversion is the synthesis of GPIs, catalyzed by CTP:phosphatidate cytidylyltransferase. This is the rate limiting step in the synthesis of GPIs(4,5)P₂ (Scheme 1). This step takes place in the endoplasmic reticulum and not in the plasma membrane. The enzyme exhibits some specificity for 1-stearoyl-2-arachidonoyl-phosphatidic acid as substrate under certain *in vitro* conditions (27). However, in order to explain the large increase we observe in arachidonoyl content in going from GPA to GPIs(4,5)P₂ (Table 1) there must be some segregation of arachidonoyl-GPA from other forms of GPA. In other words, the lipids involved in inositol cycling must be segregated from other lipids. There are two membranes involved in this process, the endoplasmic reticulum and the plasma membrane (Scheme 1). The process starts in the plasma membrane, as a consequence of hormone stimulation, activating a GPIs(4,5)P₂-specific phospholipase C that will convert an arachidonoyl-rich GPIs(4,5)P₂ to an arachidonoyl-rich DAG. The arachidonoyl-rich DAG is phosphorylated preferentially by DGK ϵ to form an arachidonoyl-rich GPA. The arachidonoyl-GPA must then be selectively transformed into GPIs lipids. This requires the transfer of GPA transfer between the two membranes, a process that is not dependent on soluble lipid carriers or on vesicular transport (28) but may occur at membrane contact sites formed between the plasma membrane and endoplasmic reticulum (29). There is evidence in yeast that such contact sites are enriched in synthases for GPSer and GPIs lipids (31). The close approach of these two membranes is also indicated by the fact that the tyrosine phosphatase, PTP-1B, in the endoplasmic reticulum acts on protein substrates of the plasma membrane (32). A possible explanation of our results is that DGK ϵ at these contact sites would preferentially transfer arachidonoyl-containing GPA. This transfer occurs more readily in the presence of DGK ϵ since the increase in arachidonoyl content between GPA and GPIs lipids is greater for the WT cells than for the KO cells. The process may also involve the DGK ϵ that is present in the endoplasmic reticulum (not shown in Scheme 1) (30), possibly at contact sites where it can directly transfer the arachidonoyl-containing GPA to GPIs synthetase, also known to be present at contact sites (31). We find that the ratio of GPA to GPIs is higher for DGK ϵ KO cells than for either WT or DGK α KO cells (Fig. 6). This is consistent with the suggested promotion of GPA transfer between membranes by DGK ϵ . In the DGK ϵ -KO cells transfer of GPA to the endoplasmic reticulum is slower and hence the concentration of this lipid increases, resulting in the change in the observed GPA/GPIs ratio. The transferred GPA would then be converted into arachidonoyl-rich GPIs and returned to the plasma membrane. The cycle would also be driven by GPA itself since this lipid regulates the transcription of GPIs synthase as well as being an allosteric regulator of type I phosphatidylinositol phosphate kinase (33). Once formed, it has been found that the GPIsP_n do not undergo remodeling (34). Thus a combination of the selective phosphorylation of arachidonoyl-DAG by DGK ϵ together with the preferential transfer of arachidonoyl-GPA from the plasma membrane to the endoplasmic reticulum, in which DGK ϵ participates in a non-catalytic role, determines the enrichment of GPIs(4,5)P₂ with arachidonoyl groups. A fraction of the arachidonoyl chains are also transferred to other phospholipids, resulting in some arachidonoyl-enrichment of several lipid classes (Fig. 2), but the enrichment of the GPIsP_n lipids is clearly the greatest. Thus, in addition to its arachidonoyl specificity in forming GPA, DGK ϵ must also have a non-catalytic role in increasing the arachidonoyl content of GPIsP_n.

The role of DGK α is quite different. This isoform causes little change in the acyl chain composition of any class of phospholipid, including the GPIsP_n (Fig. 7) or the DAG. This result is consistent with the lack of acyl chain specificity observed when DGK α is tested in *in vitro* DAG kinase assays. However, the DAG content of DGK α KO cells increases significantly (Fig. 8). Thus, unlike DGK ϵ , DGK α plays an important role in regulating the total DAG in the

cells since the absence of this isoform in DGK α KO cells leads to an increase in the DGK substrate, DAG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.
RT-PCR detection of mRNA for **DGK α** and actin in **DGK α** WT and KO cells and of mRNA for **DGK ϵ** and actin in **DGK ϵ** WT and KO cells.

Fig. 2.
Lipid profiles for the six major glycerophospholipid classes. DGK ϵ -knockout and WT cells have statistically significant differences in arachidonate containing lipids.

Fig. 3.

Lipid profiles for two major phosphatidylinositol lipids from triplicate samples of WT and KO cells. DGK ϵ -knockout and WT cells have statistically significant differences in arachidonate containing 38:4 GPIs. WT 38:4 GPIs comprises a higher percentage of the GPIs lipid class than does DGK ϵ -knockout 38:4 GPIs. The opposite trend is observed for 36:2 GPIs. The data shown are from one of three representative experiments performed. All three experiments had statistically significant differences in lipid profiles similar to those shown.

Fig. 4. Phosphatidylinositol polyphosphates mass spectral region of KO and WT mouse fibroblast extracts. DGK ϵ -knockout leads to a dramatic increase in non-arachidonate containing lipid species.

Fig. 5.

Lipid profiles for two major phosphatidylcholine lipids from one experiment (triplicate samples of WT and KO). DGK ϵ -knockout and WT cells have statistically significant differences in arachidonate containing 38:4 GPCCho. WT 38:4 GPCCho comprises a higher percentage of the GPCCho lipid class than does DGK ϵ -knockout 38:4 GPCCho. The opposite trend is observed for 36:2 GPCCho. Two additional experimental sets were also analyzed (both of which had statistically significant differences in lipid levels similar to those shown).

Fig. 6. Ratio of GPA to GPIs lipids in WT, DGK α , and DGK ϵ knockout cells. The DGK ϵ GPA/GPIs ratio was higher than that of the WT samples. Knockout of DGK α led to a decrease in GPA/GPIs compared to the WT cells.

Fig. 7.

Lipid profiles for the major phosphatidylinositol lipids. DGK α -knockout and WT cells have virtually no statistically significant differences in any GPIns lipids. The same is true for almost all glycerophospholipids.

Fig. 8.

Lipid profiles for the major diacylglycerol lipids. DGK α -knockout and WT cells had statistically significant differences in multiple DAG lipids. No significant differences were observed in the DGK ϵ -knockout experiments.

Scheme 1.

Table 1
Percent of Lipid with Arachidonoyl Chains in DGK ϵ Cells

Lipid	% PL ^a	KO	WT
DAG	-	3	3
GPA	1	11	13
GPInsP _n	5–10	38	48

^a Approximate % of total lipid for each of the phospholipid classes taken from values given in Gennis (35). The DAG concentration in a non-stimulated cell is expected to be below 1%. KO and WT columns indicate the % of each lipid class found to contain arachidonic acid.