The α Isoform of Diacylglycerol Kinase Exhibits Arachidonoyl Specificity with Alkylacylglycerol†

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ABSTRACT: We compared the diacylglycerol kinase (DGK) catalyzed phosphorylation of 1-*O*-hexanoyl-2-oleoylglycerol (HOG) with 1-*O*-hexanoyl-2-arachidonoylglycerol (HAG). We assayed the activity of DGKα and DGKζ using a liposomal-based assay system. Liposomal assays show that the DGKα and, to a lesser extent, DGKζ preferentially act on substrates containing an arachidonoyl group when this group is incorporated into alkylacylglycerols. The activity of DGKα was 82 times greater with HAG compared to HOG. DGKζ is 10 times more active in catalyzing the phosphorylation of HAG compared to HOG. Although diacylglycerols were better substrates for both DGKα and DGKζ than the alkylacylglycerols, no specificity was exhibited for arachidonoyl-containing diacylglycerols. However, this specificity for HAG over HOG is modulated by the phospholipid composition of the liposome. Addition of cholesterol and/or phosphatidylethanolamine partially reduces the substrate selectivity. We also analyzed the kinetic constants for the phosphorylation of both diacylglycerol and 1-alkyl-2-acylglycerol catalyzed by the α, ϵ, or ζ isoforms using a soluble Triton mixed micelle system. We found that all three isoforms of DGK can phosphorylate 1-alkyl-2-acylglycerols but generally at a lower rate than for the corresponding diacylglycerol. The specificity of DGKϵ for diacylglycerols containing an arachidonoyl group was retained when the ester group in the C-1 position is replaced with an ether linkage. In contrast, DGKα and, to a lesser extent, DGKζ had greater specificity for arachidonoyl-containing 1-alkyl-2-acylglycerols than for arachidonoyl-containing diacylglycerols. This demonstrates that the acyl chain specificity is affected by the structure of the lipid headgroup.

There are at least nine different isoforms of mammalian diacylglycerol kinase (DGK)[1] which have been separated into five classes on the basis of their different structures and properties (*1*−*5*). DGKζ is a 117 kDa protein that can translocate to the nucleus, where it can reduce the amount of nuclear diacylglycerol (DAG) resulting in the attenuation of cell growth (*6*, *7*). This isoform has also been shown to be abundant in the brain and to be present in regions undergoing apoptosis (*8*). The activity of DGKζ is regulated by phosphorylation (*9*, *10*). In contrast, DGKα, an 83 kDa enzyme, promotes cell proliferation (*11*), translocates to the plasma membrane (*12*), and is activated by calcium (*13*) and by the lipid products of phosphatidylinositol-3-kinase (*14*). Both DGKζ and DGKα prevent Ras activation by lowering DAG levels (*15*−*17*). DGKϵ has a molecular mass of 64 kDa and has been shown to regulate seizure susceptibility and long-term potentiation (*18*).

The ϵ isoform of DGK exhibits specificity for DAG containing an arachidonoyl chain (*19*, *20*) or other polyunsaturated acyl chains (*21*). Specificity for arachidonoyl-containing substrates has also recently been found with a form of DGK from *Arabidopsis* (*22*). Specificity for substrates containing an arachidonoyl moiety may have particular physiological importance. Activation of certain isoforms of phospholipase C as a consequence of hormone stimulation results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate, a phospholipid whose major acyl chains are stearoyl and arachidonoyl groups (*23*, *24*).

Little information is available regarding the substrate specificity among different forms of DGKs for the headgroup structures of lipids. In this study we have compared substrates with ester vs ether linkage at the *sn*-1 of glycerol. The diacylglycerols are derived from diacyllipids, while the ether-containing glycerol derivatives arise from alkyl ester lipids including plasmalogens. Plasmalogens comprise a significant fraction of the phosphatidylcholine and phosphatidylethanolamine in cell membranes (*25*). Of particular importance for many physiologic events may be arachidonoyl-containing alkyllipids present in raft fractions. It has recently been estimated that over one-third to one-half of the phospholipids

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[1] Abbreviations: AAG, 1-alkyl-2-acyl-*sn*-glycerol; BHT, butylated hydroxytoluene; DAG, diacylglycerol; DGK, diacylglycerol kinase; DOG, 1,2-dioleoyl-*sn*-glycerol; DOPC, 1,2-dioleoylphosphatidylcholine; DOPE, 1,2-dioleoylphosphatidylethanolamine; DOPS, 1,2-dioleoylphosphatidylserine; DTT, dithiothreitol; HAG, 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerol; HOG, 1-*O*-hexadecyl-2-oleoyl-*sn*-glycerol; LUV, large unilamellar vesicles; NP-40, nonaethylene glycol octaphenyl ether; PA, phosphatidic acid; PMSF, phenylmethanesulfonyl fluoride; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol.

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in the cytoplasmic face of the raft fraction are arachidonoyl-containing alkyllipids (*26*). These phospholipids give rise to ether-linked diradylglycerol species in response to the stimulation of some cells with certain ligands (*25*). The chemical structure of 1-alkyl-2-acylglycerol (AAG) is very closely related to DAG, but few studies have evaluated whether these lipids are substrates for DAG kinases. The potential physiological importance of the difference between alkyl- and acyl-linked lipids is given, for example, by the recent finding that acyl and alkyl ether linked lysophosphatidic acids produce different responses in human platelets (*27*). In a study with DGK, Jones et al. (*28*) demonstrated that DGKα could phosphorylate AAG, although the acyl chain specificity of this reaction was not investigated. This is an important question because stimulation of lymphocytes with interleukin-2 leads to the phosphorylation of a preexisting pool of AAG (*28*), suggesting that these alkyllipids or their phosphorylated counterparts may have important signaling functions. Both DAG and AAG have been shown to prime the respiratory burst in human neutrophils in vitro (*29*). AAGs are produced in cells from phosphatidylcholine by activation of phospholipase D followed by action of a phosphatidate phosphohydrolase (*30*, *31*). It would not be surprising if DAG kinases other than DGKα could phosphorylate AAGs because it has been commonly observed that enzymes often do not discriminate between lipid substrates that have ether linkages compared with similar substrates with only acyl groups (*25*). If AAGs are substrates for different DGK isoforms, it would be important to determine if the specificity for acyl chains in the substrate is the same as that found for DAG.

EXPERIMENTAL PROCEDURES

Materials. Lipids, including the DGK substrates, were purchased from Avanti Polar Lipids (Alabaster, AL). Histone HI was from GIBCO/BRL (Grand Island, NY); ATP was the disodium salt, SigmaUltra grade (Sigma, St. Louis, MO). [γ-^{32}P]ATP was from ICN. Other chemicals were of the purest grade available. Doubly distilled water was used for all solutions.

Enzyme Preparations. cDNAs encoding either DGKα, DGKϵ, or DGKζ were cloned into BacPAK6 (Clontech), and baculovirus stocks were generated using the BakPAK system (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Sf21 cells were infected with the virus stocks, and then the cells were harvested 72 h later in 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 1 mM PMSF, 0.5 mM sodium orthovanadate, and 10 mg/mL each of leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor. They were allowed to lyse for 10 min and then centrifuged at low speed to remove debris. The particulate material remaining in the supernate was then pelleted. This pellet was then sonicated for 5 min before use with alternating cycles of 30 s on or off (*32*) and either used directly in the Triton micelle assay (see below) or used following dilution of 10 μL of the sonicated suspension into 90 μL of 2 M KCl solution that was buffered with Tris-HCl at pH 8.0. Soluble proteins for use in liposome-based assays were separated by centrifugation (100000*g*, 30 min, 20 °C). The fraction of total protein recovered in the salt-extracted preparation was 78 ± 4% and 74 ± 9% for DGKα and DGKζ, respectively. Although the salt extraction procedure did not substantially increase the specific activity of the enzyme, it did convert the DGK activity to a water-soluble form that resulted in greater and more reproducible activity in liposome-based assays. Control enzyme was prepared by mock-infecting the Sf21 cells.

Preparation of Liposomes. Lipids were codissolved in chloroform/methanol (2:1 v/v). The solvent was then evaporated under a stream of nitrogen with constant rotation of a test tube so as to deposit a uniform film of lipid over the bottom third of the tube. The last traces of solvent were removed by placing the tube under high vacuum for at least 2 h. The lipid film was then hydrated to a final concentration of 19 mM in assay buffer (75 mM Tris-HCl, 7.5 mM $MgCl_2$, and 150 mM NaCl, pH 8.0). Large unilamellar vesicles (LUVs) were made from this suspension by five freeze−thaw cycles and extruded 10 times through two 0.1 μm pore polycarbonate filters.

DGK Activity Assay Using Liposomes. Aliquots of LUVs (50 μL) were diluted to a final volume of 200 μL, containing 4.75 mM lipids, 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 5 μg/mL histones, and 5 μL of 10×-diluted, salt-extracted enzyme (~7.5 μg of protein) in the presence of either $CaCl_2$ or EGTA as indicated. In the results presented, a Ca^{2+} concentration of 1 mM was used. We also determined the calcium concentration dependence of the rate of phosphorylation. We found that maximal activity was reached at a concentration below 10 μM (not shown). We present the results obtained with a high calcium concentration. These results are similar to those obtained at 10 μM Ca^{2+}, but they exhibit smaller variability among assays. The reaction was initiated by the addition of 20 μL of 5 mM [γ-^{32}P]ATP (40 μCi/mL). After incubation at 25 °C for 2 min, the reaction was terminated with 2 mL of $CHCl_3$/CH_3-OH (1:1) containing 0.25 mg/mL dicetyl phosphate. The chloroform phase was washed three times with 2 mL of 1% $HClO_4$ and 0.1% H_3PO_4 in H_2O/methanol (7:1 v/v). The volume of the final $CHCl_3$ phase was 0.80 mL. A 400 μL aliquot of the organic phase was dried under N_2, and the incorporation of ^{32}P into PA was determined by Cerenkov counting in a Beckman-Coulter scintillation counter. Controls were run without the addition of enzyme or with the addition of mock-infected Sf21 cell lysates. In both cases, the counts remaining in the organic phase were only slightly above the background level. The DGK activity measured with mock-infected cells was subtracted from the values obtained using cells overexpressing one of the isoforms of DGK. The production of PA was linear with time over 3 min.

Each determination of activity is the average activity of three replicates. The errors given in the tables represent the standard deviation. Each entry in the table represents assays carried out on a single day. Replicate experiments carried out on different days show similar effects for different conditions, but the absolute activity obtained on each day is dependent on the amount of protein extracted during centrifugation and exhibits a greater variability in absolute activity. The relative activities of the different isoforms of DGK used in this work should not be compared with one another since they each likely correspond to different expression levels.

Triton Micelle-Based Assay of DGK Activity. Our assay was adapted from the procedure described by Walsh et al. (*20*). Briefly, for each assay, 4 μL of a DAG or AAG stock

Table 1: Relative Rates of Phosphorylation of HOG and HAG by DGKα or DGKζ

isoform	liposome composition[c]	Ca^{2+} [d]	activity[a] HOG	activity[a] HAG	blank activity[b] HOG	blank activity[b] HAG	ratio[e]
α	65% DOPC−30% DOPS	+	0.22 ± 0.05	18 ± 4	0.014 ± 0.001	0.016 ± 0.001	82
α	15% DOPC−30% DOPS−30% DOPE−20% cholesterol	+	0.20 ± 0.02	5.0 ± 0.8	0.0081 ± 0.0007	0.0112 ± 0.0009	25
α	15% DOPC−30% DOPS−30% DOPE−20% cholesterol	−	0.4 ± 0.05	2.3 ± 0.2	0.011 ± 0.004	0.012 ± 0.002	6
ζ	65% DOPC−30% DOPS	+	0.69 ± 0.03	7.3 ± 0.9	0.009 ± 0.003	0.008 ± 0.001	11
ζ	65% DOPC−30% DOPS	−	0.67 ± 0.04	6.9 ± 1.3	0.0068 ± 0.0005	0.0075 ± 0.003	10
ζ	20% DOPC−35% DOPS−40% DOPE	+	0.8 ± 0.1	1.27 ± 0.03	0.007 ± 0.001	0.0067 ± 0.0003	1.6
ζ	20% DOPC−35% DOPS−40% DOPE	−	0.89 ± 0.08	3.6 ± 0.3	0.011 ± 0.005	0.010 ± 0.001	4

[a] Initial rate of substrate phosphorylation. Units of nanomoles of PA formed per minute. Activity represents the average of three replicates. Errors represent the standard deviation among the three replicates under each condition. [b] Control using mock-transfected cells. [c] Lipid composition of LUVs, not including substrate that is present at 5 mol %. [d] Presence (+) or absence (−) of 1 mM Ca^{2+}. [e] Ratio of activity against HAG/HOG.

solution (40 mM in 1:1 $CHCl_3/CH_3OH$, stored at −20 °C under argon) together with any other lipid component being used in the assay was deposited on the walls of a glass test tube by solvent evaporation under a stream of nitrogen and then placed under vacuum for 2 h to remove the last traces of solvent. The lipid was hydrated with 50 μL of buffer made at four times the concentration used in the final assay (60 mM Triton X-100, 200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 20 mM $MgCl_2$, 4 mM EGTA, and 30 μg/mL BHT), 2 μL of 100 mM DTT, 1 μL of 1 mg/mL histone, and 5 μL of cell lysate either from cells transfected with one of the DGK isoforms or from mock-transfected cells. The final volume was 180 μL. The reaction was initiated by adding 20 μL of 5.0 mM [γ-^{32}P]ATP (75 μCi/mL). The final assay mixture contained 15 mM Triton X-100, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.75 μg of BHT, 1 mM DTT, 5 μg/mL histone, the enzyme preparation, and 0.80 mM DAG or AAG. For assays with DGKζ, 5 mM DOPS was also added to the assay since it substantially enhances the activity of this isoform (*32*). The reaction was carried out for 10 min at 25 °C and was terminated by extraction of the lipid with the addition of 2 mL of $CHCl_3$/methanol (1:1 v/v) containing 0.25 mg/mL dihexadecyl phosphate. The organic phase was washed three times with 2 mL each of 1% $HClO_4$ and 0.1% H_3PO_4 in H_2O/methanol (7:1 v/v). The volume of the final $CHCl_3$ phase was 0.80 mL. A 0.50 mL aliquot of the organic phase was dried under N_2, and the incorporation of ^{32}P into PA was determined by Cerenkov counting. Controls were run without the addition of enzyme or with the addition of mock-transfected cell lysates. In both cases, the counts remaining in the organic phase were only slightly above the background level. The DGK activity measured with mock-transfected cells was subtracted from the values obtained using cells overexpressing one of the isoforms of DGK. The production of PA was linear with time over 10 min.

The assays were done in triplicate and the results presented with errors showing the standard deviation of the mean for one particular experiment. Each experiment was independently repeated at least twice. The day-to-day variations using the same enzyme preparation and the same lipids were not much greater than those for an individual experiment.

Kinetic Analysis of the Micelle-Based Assay of DGK Activity. Kinetic analysis was performed in Triton micelles, as described above, using a range of DAG/AAG substrate concentrations from 0.05 to 1.0 mM and maintaining ATP constant at 0.5 mM. A kinetic analysis was performed on each of the DGK isoforms and for each of the lipid substrates. The Michaelis−Menten constants, V_{max} and K_m, were evaluated using Lineweaver−Burk plots (i.e., $1/v$ vs $1/[S]$). For assays with DGKα, 2.5 mM $CaCl_2$ and 3.5 mM DOPS were also presented. Microsoft Excel was used in determining the Lineweaver−Burk plots, and the corresponding errors of V_{max} and K_m were obtained by the data analysis function along with the line-residual plot.

RESULTS

Liposomal Assays of DGK Activity. Liposomal assays allow for the use of lipids in bilayer, rather than micellar, form. This makes the system closer to that of a biological membrane. We have added DOPS to the liposomes since it enhances the activity of some of the isoforms of DGK and it is a major component of the lipid composition of the cytoplasmic leaflet of the plasma membrane. We have compared a simple lipid mixture of DOPS with DOPC, the latter being an abundant lipid of the plasma membrane. Choice of DOPC also allowed us to test the effects of changing the curvature properties of the membrane by substituting DOPE or cholesterol for DOPC. All of these lipids represent major components of the cytoplasmic leaflet of the plasma membrane.

DGKα Shows Substrate Specificity for HAG over HOG in Liposomal Assays. We compared the acyl chain specificity of DGKα using two AAG substrates (Table 1). DGKα catalyzes the phosphorylation of 1-*O*-hexadecyl-2-arachidonoylglycerol (HAG) 82-fold more rapidly than 1-*O*-hexadecyl-2-oleoylglycerol (HOG).

We determined if the substrate specificity of DGKα was sensitive to the changes in the lipid composition that would modify the physical properties of the membrane as well as compared the substrate specificity in the presence and absence of Ca^{2+}. We have recently shown (*37*) that the activity of DGKα becomes independent of calcium when the liposomes contain lipids such as DOPE and cholesterol that promote negative curvature. The mechanism by which DOPE and cholesterol act is discussed in detail in ref *37*. Substrate specificity for HAG when the liposomes were enriched with 30% DOPE and 20% cholesterol, either in the presence or absence of Ca^{2+}, was still present but diminished (Table 1). Without addition of DOPE and cholesterol, the activity of DGKα was very low in the absence of calcium and is not reported. DGKα is an isoform with an EF-hand domain that is thought to augment its activity in the presence of calcium (*33*). The preferential phosphorylation of HAG by DGKα is observed in both the presence and absence of

Table 2: Comparison of DOG and HOG as Substrates

isoform	liposome composition[c]	Ca^{2+} [d]	activity[a] DOG	activity[a] HOG	blank activity[b] DOG	blank activity[b] HOG	ratio[e]
α	15% DOPC−30% DOPS−30% DOPE−20% cholesterol	+	3.4 ± 0.9	0.61 ± 0.03	0.0078 ± 0.0009	0.0063 ± 0.0013	6
ζ	20% DOPC−35% DOPS−40% DOPE	+	11 ± 2	4 ± 1	0.4 ± 0.4	0.04 ± 0.02	3
ζ	20% DOPC−35% DOPS−40% DOPE	−	16 ± 5	5 ± 1	0.065 ± 0.003	0.064 ± 0.015	3

[a] Initial rate of substrate phosphorylation. Units of nanomoles of PA formed per minute. Activity represents the average of three replicates. Errors represent the standard deviation among the three replicates under each condition. [b] Control using mock-transfected cells. [c] Lipid composition of LUVs, not including substrate that is present at 5 mol %. [d] Presence (+) or absence (−) of 1 mM Ca^{2+}. [e] Ratio of activity against DOG/HOG.

Table 3: Comparison of DOG and SAG as Substrates

isoform	liposome composition[c]	Ca^{2+} [d]	activity[a] DOG	activity[a] SAG	blank activity[b] DOG	blank activity[b] SAG	ratio[e]
α	20% DOPC−35% DOPS−40% DOPE	+	4.4 ± 0.6	4.9 ± 0.8	0.015 ± 0.002	0.0145 ± 0.001	1
ζ	65% DOPC−30% DOPS	−	2.5 ± 0.3	2.5 ± 0.5	0.013 ± 0.002	0.010 ± 0.002	1
ζ	20% DOPC−35% DOPS−40% DOPE	+	6.3 ± 0.4	7.4 ± 0.9	0.015 ± 0.002	0.015 ± 0.001	1
ζ	20% DOPC−35% DOPS−40% DOPE	−	7.6 ± 0.5	8.2 ± 0.9	0.0096 ± 0.0008	0.0108 ± 0.0008	1

[a] Initial rate of substrate phosphorylation. Units of nanomoles of PA formed per minute. Activity represents the average of three replicates. Errors represent the standard deviation among the three replicates under each condition. [b] Control using mock-transfected cells. [c] Lipid composition of LUVs, not including substrate that is present at 5 mol %. [d] Presence (+) or absence (−) of 1 mM Ca^{2+}. [e] Ratio of activity against SAG/DOG.

calcium. The activity of mock-infected Sf21 cells that were used as controls is small (Table 1).

DGKζ Preferentially Acts on HAG To Convert It to Phosphatidic Acid. DGKζ also exhibits some specificity for HAG over HOG (Table 1) but not as great as in the case of DGKα. In both the presence and absence of Ca^{2+}, DGKζ exhibits about 10-fold greater activity with HAG as substrate, compared with HOG. DGKζ is not known to be activated by calcium and has no calcium-binding motif. Assays run in the presence of both 1 mM calcium and 0.5 mM EGTA gave similar results (Table 1). As with DGKα, addition of a negative curvature lipid, DOPE, lowered but did not eliminate the specificity of DGKζ for HAG over HOG (Table 1).

Comparison of Substrates DOG and HOG with DGKα and DGKζ. DAG is a better substrate for both DGKα and DGKζ than is an AAG substrate (Table 2). This is in agreement with earlier findings with mixed DGK isoforms (*34*).

Comparison of DAG Substrates DOG and SAG with DGKα and DGKζ. Both DGKα and DGKζ exhibited similar rates of phosphorylation of SAG and DOG (Table 3).

Comparison of the Kinetic Constants for DGK-Catalyzed Phosphorylation of Substrates in Triton Micelles. A Triton micelle-based assay was employed to compare the kinetics of phosphorylation of DAG and AAG substrates. This allowed for a more quantitative comparison, not dependent on the extent of extraction of the enzyme from the cell pellet. We recognize that modulation of enzyme activities by lipids is not likely to be identical in micelles and bilayers. However, since DGKε was the only isoform with known arachidonoyl specificity and a bilayer-based assay for this isoform has not yet been achieved, we felt it important to use the detergent system to be able to compare all three isoforms. We determined the Michaelis−Menten kinetic constants for the three isoforms of DGK using both DAG and AAG substrates. The K_m is given in terms of the mole fraction of substrate in the micelle. The ratio, V_{max}/K_m, is a measure of the catalytic efficiency and corresponds to the catalytic rate constant at low substrate concentration. DGKε selectively phosphorylates DAG with an arachidonate group at the *sn*-2 position as a consequence of a decrease in K_m for this isoform (*20*). Our value for the K_m of SAG is in reasonable agreement with the value found for the arachidonoyl-specific isoform from testes (*20*), 2.4 mol %, but our K_m for DOG is lower than the value previously found for 1-palmitoyl-2-oleoylglycerol (*20*). The higher value for K_m determined for 1-palmitoyl-2-oleoylglycerol was obtained using a micelle system containing the cationic detergent OTAB. Although the presence of OTAB did not alter the K_m determined for SAG (*20*), it is possible that it contributed to the higher value observed with 1-palmitoyl-2-oleoylglycerol. Hence there are two differences between the work reported here and the values in the literature: one being the difference in the chemical nature of the non-arachidonoyl-containing substrate and the other being the nature of the detergent assay as well as the difference in the source of the enzyme. We tested whether DGKε also phosphorylated AAG. Although the AAGs were poorer substrates than the corresponding DAG for DGKε, the higher catalytic rate with this isoform for arachidonoyl-containing lipids is maintained, as a consequence of differences in V_{max} (Table 4). DGKα catalyzes the phosphorylation of SAG at comparable rates to DOG. However, DGKα had much higher activity toward AAG when it contained an arachidonate group (HAG). In addition, this was the only DGK isoform that phosphorylated an AAG at a higher rate than for the corresponding DAG. DGKζ shows little discrimination among the four lipid substrates tested. The Lineweaver−Burk plot for DGKζ and SAG as substrate had a low correlation coefficient, after several attempts, and the K_m is therefore not reported for this case. These data indicate that the 1-alkyl-2-acylglycerols are substrates for all three DGK isoforms, α, ε, and ζ. Our results also demonstrate that the acyl components of the AAGs can significantly affect their suitability as substrates for DGK and that the higher catalytic rate found for arachidonoyl-containing lipids is also dependent on the nature of the headgroup.

Table 4: Michaelis−Menten Kinetic Constants for DGK Assays in Triton Micelles[a]

DGK isoform	substrate	K_m (mol %)	V_{max}[b]	V_{max}/K_m[b]
α-isoform	SAG	3.3 ± 0.7	9 ± 2	2.7
	DOG	3.0 ± 0.4	9 ± 1	3
	HAG	0.8 ± 0.1	3.2 ± 0.5	4
	HOG	0.97 ± 0.05	0.08 ± 0.005	0.08
ϵ-isoform	SAG	1.6 ± 0.2	3.35 ± 0.54	2.1
	DOG	2.3 ± 0.2	3.5 ± 0.3	1.5
	HAG	2.2 ± 0.4	0.12 ± 0.03	0.05
	HOG	0.87 ± 0.03	0.014 ± 0.001	0.016
ζ-isoform	SAG	ND[c]	0.40 ± 0.03	ND
	DOG	1.6 ± 0.1	0.57 ± 0.05	0.36
	HAG	1.3 ± 0.1	0.41 ± 0.03	0.32
	HOG	0.64 ± 0.05	0.073 ± 0.003	0.11

[a] Micelle-based assays contained 15 mM Triton X-100 in the buffer 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, and 0.75 μg of BHT, 1 mM DTT, 0.5 mM ATP, 5 μg/mL histone, and 5 μL of cell lysate. Each assay used a range of substrate concentration of 0.05−1.0 mM in Triton micelles; 3.5 mM DOPS and 2.5 mM $CaCl_2$ were added to the reactions involving DGKα. Formation of the product, phosphatidic acid (PA), was determined after extraction by the incorporation of ^{32}P. The counts were corrected for the DGK activity of noninfected cells. [b] Values are V_{max} are relative values since the amount of enzyme in the cell preparations is not known. The values of V_{max} and of V_{max}/K_m can be compared to one another for a particular isoform but not between one isoform and another. [c] ND = not determined.

DISCUSSION

We have been able to transfer the α and ζ isoforms of DGK to liposomes to establish an enzyme activity assay based on using bilayer membranes. We have also assayed these isoforms in Triton micelles to compare them with DGKϵ and to obtain more quantitative comparisons. The kinetic constants that we obtained with this analysis demonstrate that AAGs are poorer substrates for all of the DGK isoforms compared with DAG, primarily because of differences in V_{max} (Table 4). This is in good agreement with findings using DGK from rabbit brain with an octyl glucoside based mixed micelle assay (*34*). The replacement of the *sn*-1 ester for an ether bond caused more dramatic changes when we examined DGKα either in liposomes (Table 1) or in Triton micelles (Table 4). This isoform exhibited the largest difference in rate of phosphorylation between HAG and HOG of those tested. These results demonstrate that the substrate selectivity of DGKs depends on whether they are phosphorylating DAG or AAG. Our observations also suggest that this selectivity for different molecular species of DAG or AAG may not simply be due to direct recognition of the acyl chain. The fact that the increased rate of phosphorylation of HAG over HOG is a consequence of an increase in V_{max}, rather than a decrease in K_m, also suggests that the difference is not caused by a change in affinity of the enzyme for the substrate.

The liposome-based assays more closely resemble the conditions of a cell membrane. They also show that the substrate selectivity is modulated by the nature of the surrounding lipids and that liposomes comprised of lipids with less hydration and more intrinsic negative curvature reduce the selectivity of DGKα for HAG over HOG (Table 1).

Jones et al. (*28*) characterized the molecular species of 1-alkyl-2-acylglycerols in CTLL-2 cells and did not detect any polyunsaturated AAGs. They also noted that the molecular species of AAGs closely correlated with that of phosphatidylcholines. The lack of detectable polyunsaturated AAGs suggests that our observations may not be physiologically relevant. However, other investigators have noted that in neutrophils 66% of arachidonoyl moieties in phosphatidylcholines are in 1-alkyl-2-arachidonoyl species (*35*) and in leukocytes and monocytes the alkylphosphatidylcholines also have a high arachidonate content, implying that arachidonate AAGs likely exist in some cell types (*36*). This new specificity for arachidonoyl-containing AAGs, which are not ubiquitous, may represent a mechanism to activate signaling cascades or to enrich AAGs and possibly alkylphosphatidylcholines with arachidonate only in certain cell types.

Several enzymes that act on DAG do not discriminate between diacyl and alkylacyl lipids. Consistent with this, we found that the three isoforms of DGK could phosphorylate AAG in a Triton micelle-based assay. However, except for DGKα, the activity toward AAG was slightly lower when compared with the corresponding DAG. Consistent with previous evidence demonstrating that DGKϵ selectively phosphorylates arachidonoyl DAG compared to other diacylglycerols (*19*−*21*), we found that its specificity for arachidonoyl-containing, alkyllipids was maintained (Table 4). In addition, we demonstrate that DGKα has even greater specificity for arachidonoyl-containing AAG.

REFERENCES

1. Van Blitterswijk, W. J., and Houssa, B. (2000) Properties and functions of diacylglycerol kinases, *Cell Signalling 12*, 595−605.
2. Topham, M. K., and Prescott, S. M. (1999) Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions, *J. Biol. Chem. 274*, 11447−11450.
3. Kanoh, H., Yamada, K., and Sakane, F. (2002) Diacylglycerol kinases: emerging downstream regulators in cell signaling systems, *J. Biochem. (Tokyo) 131*, 629−633.
4. Topham, M. K., and Prescott, S. M. (2002) Diacylglycerol kinases: regulation and signaling roles, *Thromb. Haemostasis 88*, 912−918.
5. Luo, B., Regier, D. S., Prescott, S. M., and Topham, M. K. (2004) Diacylglycerol kinases, *Cell Signalling 16*, 983−989.
6. Topham, M. K., Bunting, M., Zimmerman, G. A., McIntyre, T. M., Blackshear, P. J., and Prescott, S. M. (1998) Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta, *Nature 394*, 697−700.
7. Hozumi, Y., Ito, T., Nakano, T., Nakagawa, T., Aoyagi, M., Kondo, H., and Goto, K. (2003) Nuclear localization of diacylglycerol kinase zeta in neurons, *Eur. J. Neurosci. 18*, 1448−1457.
8. Ding, L., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1998) The cloning and developmental regulation of murine diacylglycerol kinase zeta, *FEBS Lett. 429*, 109−114.
9. Abramovici, H., Hogan, A. B., Obagi, C., Topham, M. K., and Gee, S. H. (2003) Diacylglycerol kinase-zeta localization in skeletal muscle is regulated by phosphorylation and interaction with syntrophins, *Mol. Biol. Cell 14*, 4499−4511.
10. Luo, B., Prescott, S. M., and Topham, M. K. (2003) Protein kinase C{alpha} phosphorylates and negatively regulates diacylglycerol kinase {zeta}, *J. Biol. Chem. 278*, 39542−39547.
11. Flores, I., Casaseca, T., Martinez, A., Kanoh, H., and Merida, I. (1996) Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation, *J. Biol. Chem. 271*, 10334−10340.
12. Sanjuan, M. A., Jones, D. R., Izquierdo, M., and Merida, I. (2001) Role of diacylglycerol kinase alpha in the attenuation of receptor signaling, *J. Cell Biol. 153*, 207−220.
13. Sakane, F., Yamada, K., Imai, S., and Kanoh, H. (1991) Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation, *J. Biol. Chem. 266*, 7096−7100.

14. Cipres, A., Carrasco, S., Merino, E., Diaz, E., Krishna, U. M., Falck, J. R., Martinez, A., and Merida, I. (2003) Regulation of diacylglycerol kinase alpha by phosphoinositide 3-kinase lipid products, *J Biol. Chem. 278*, 35629−35635.
15. Zhong, X. P., Hainey, E. A., Olenchock, B. A., Zhao, H., Topham, M. K., and Koretzky, G. A. (2002) Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta, *J. Biol. Chem. 277*, 31089−31098.
16. Topham, M. K., and Prescott, S. M. (2001) Diacylglycerol kinase zeta regulates Ras activation by a novel mechanism, *J. Cell Biol. 152*, 1135−1143.
17. Jones, D. R., Sanjuan, M. A., Stone, J. C., and Merida, I. (2002) Expression of a catalytically inactive form of diacylglycerol kinase alpha induces sustained signaling through RasGRP, *FASEB J. 16*, 595−597.
18. De Turco, E. B. R., Tang, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., Taketomi, A., Prescott, S. M., and Bazan, N. C. (2001) Diacylglycerol kinase ϵ regulates seizure susceptibility and long-term potentiation through arachidonoyl-inositol lipid signaling, *Proc. Natl. Acad. Sci. U.S.A. 98*, 4740−4745.
19. Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates, *J. Biol. Chem. 271*, 10237−10241.
20. Walsh, J. P., Suen, R., Lemaitre, R. N., and Glomset, J. A. (1994) Arachidonoyl-diacylglycerol kinase from bovine testis. Purification and properties, *J. Biol. Chem. 269*, 21155−21164.
21. Pettitt, T. R., and Wakelam, M. J. (1999) Diacylglycerol kinase epsilon, but not zeta, selectively removes polyunsaturated diacylglycerol, inducing altered protein kinase C distribution in vivo, *J. Biol. Chem. 274*, 36181−36186.
22. Gomez-Merino, F. C., Brearley, C. A., Ornatowska, M., Abdel-Haliem, M. E. F., Zanor, M. I., and Mueller-Roeber, B. (2004) AtDGK2, a novel diacylglycerol kinase from *Arabidopsis thaliana*, phosphorylates 1-stearoyl-2-arachidonoyl-*sn*-glycerol and 1,2-dioleoyl-*sn*-glycerol and exhibits cold-inducible gene expression, *J. Biol. Chem. 279*, 8230−8241.
23. Gaudette, D. C., Aukema, H. M., Jolly, C. A., Chapkin, R. S., and Holub, B. J. (1993) Mass and fatty acid composition of the 3-phosphorylated phosphatidylinositol bisphosphate isomer in stimulated human platelets, *J. Biol. Chem. 268*, 13773−13776.
24. Reddy, T. S., and Bazan, N. G. (1987) Arachidonic acid, stearic acid, and diacylglycerol accumulation correlates with the loss of phosphatidylinositol 4,5-bisphosphate in cerebrum 2 s after electroconvulsive shock: complete reversion of changes 5 min after stimulation, *J. Neurosci. Res. 18*, 449−455.
25. Nagan, N., and Zoeller, R. A. (2001) Plasmalogens: biosynthesis and functions, *Prog. Lipid Res. 40*, 199−229.
26. Pike, L. J., Han, X., Chung, K. N., and Gross, R. W. (2002) Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis, *Biochemistry 41*, 2075−2088.
27. Tokumura, A., Sinomiya, J., Kishimoto, S., Tanaka, T., Kogure, K., Sugiura, T., Satouchi, K., Waku, K., and Fukuzawa, K. (2002) Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids, *Biochem. J. 365*, 617−628.
28. Jones, D. R., Pettitt, T. R., Sanjuan, M. A., Merida, I., and Wakelam, M. J. (1999) Interleukin-2 causes an increase in saturated/monounsaturated phosphatidic acid derived from 1,2-diacylglycerol and 1-O-alkyl-2- acylglycerol, *J. Biol. Chem. 274*, 16846−16852.
29. Bass, D. A., McPhail, L. C., Schmitt, J. D., Morris-Natschke, S., McCall, C. E., and Wykle, R. L. (1988) Selective priming of rate and duration of the respiratory burst of neutrophils by 1,2-diacyl and 1-O-alkyl-2-acyl diglycerides. Possible relation to effects on protein kinase C, *J. Biol. Chem. 263*, 19610−19617.
30. Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W., and Siegel, M. I. (1989) Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction, *J. Biol. Chem. 264*, 17069−17077.
31. Chabot, M. C., McPhail, L. C., Wykle, R. L., Kennerly, D. A., and McCall, C. E. (1992) Comparison of diglyceride production from choline-containing phosphoglycerides in human neutrophils stimulated with N-formylmethionyl-leucylphenylalanine, ionophore A23187 or phorbol 12-myristate 13-acetate, *Biochem. J. 286* (Part 3), 693−699.
32. Thirugnanam, S., Topham, M. K., and Epand, R. M. (2001) Physiological implications of the contrasting modulation of the activities of the ϵ and ζ isoforms of diacylglycerol kinase, *Biochemistry 40*, 10607−10613.
33. Yamada, K., Sakane, F., Matsushima, N., and Kanoh, H. (1997) EF-hand motifs of alpha, beta and gamma isoforms of diacylglycerol kinase bind calcium with different affinities and conformational changes, *Biochem. J. 321* (Part 1), 59−64.
34. Ford, D. A., and Gross, R. W. (1990) Differential metabolism of diradyl glycerol molecular subclasses and molecular species by rabbit brain diglyceride kinase, *J Biol. Chem. 265*, 12280−12286.
35. Chilton, F. H., and Connell, T. R. (1988) 1-ether-linked phosphoglycerides. Major endogenous sources of arachidonate in the human neutrophil, *J. Biol. Chem. 263*, 5260−5265.
36. Prescott, S. M., Zimmerman, G. A., Stafforini, D. M., and McIntyre, T. M. (2000) Platelet-activating factor and related lipid mediators, *Annu. Rev. Biochem. 69*, 419−445.
37. Fanani, M. L., Topham, M. K., Walsh, J. P., and Epand, R. M. (2004) Lipid modulation of the activity of diacylglycerol kinase alpha- and zeta-isoforms: Activation by phosphatidylethanolamine and cholesterol, *Biochemistry 43*, in press.

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