Substrate Chirality and Specificity of Diacylglycerol Kinases and the Multisubstrate Lipid Kinase†

Richard M. Epand,*,‡ Yulia V. Shulga,‡ Heath C. Timmons,§ Alexandra L. Perri,‡ Jitendra D. Belani,§ Kirishanth Perinpanathan,‡ Laura Beth Johnson-McIntire,|| Sandra Bajjalieh,|| Armela O. Dicu,‡ Cynthia Elias,⊥ Scott D. Rychnovsky,§ and Matthew K. Topham@

Department of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada, Department of Chemistry, 1102 Natural Sciences 2, University of California, Irvine, California 92697-2025, Department of Pharmacology, University of Washington, D429 HSB, Box 357280, Seattle, Washington 98195-7280, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, Québec H4P 2R2, Canada, and Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112

Received August 7, 2007; Revised Manuscript Received October 4, 2007

ABSTRACT: The α, ζ, and ϵ isoforms of diacylglycerol kinase exhibit a high degree of stereospecificity in the phosphorylation of diacylglycerol. In comparison, a multiple lipid kinase, MuLK, shows much less stereospecificity, phosphorylating 1,2-dioleoylglycerol only ∼2−3 times more rapidly than 2,3-dioleoylglycerol. The α and ζ isoforms of diacylglycerol kinase are inhibited by 2,3-dioleoylglycerol, but not the more substrate-selective ϵ isoform. The inhibition by 2,3-dioleoylglycerol is uncompetitive. This corresponds to a kinetic scheme in which the inhibitor can bind to the enzyme−substrate complex, but not to the free enzyme. Our data indicate that despite their similar structures, 1,2-dioleoylglycerol and 2,3-dioleoylglycerol do not compete for the active site of these three isoforms of diacylglycerol kinase. We suggest that the 2,3-dioleoylglycerol binds to a site on the α and ζ isoforms of diacylglycerol kinase that is exposed as a consequence of the substrate binding to the active site. The chiral specificity of these enzymes thus mimics the substrate specificity, with MuLK being the least selective and the ϵ isoform of diacylglycerol kinase exhibiting the greatest selectivity.

Mammalian diacylglycerol kinases (DGKs)[1] make up a family of enzymes that catalyze the production of phosphatidic acid from diacylglycerol (DAG) and ATP. These enzymes are believed to have important roles in DAG signaling (*1*, *2*). Another lipid kinase that phosphorylates diacylglycerol is the multisubstrate lipid kinase (MuLK) (*3*, *4*). This enzyme resembles bacterial DGK in having less specificity for the lipid substrate it phosphorylates (*3*), although its sequence is more homologous to those of mammalian forms of DGK, and it is not an integral membrane protein like the bacterial DGKs (*5*). The sequence of MuLK also is significantly similar to those of sphingosine kinases. Because it catalyzes the phosphorylation of DAG at a higher rate than other lipids (*3*), it can be considered as a form of DGK.

There are only two widely utilized inhibitors of mammalian DGKs. These are 6-(2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl)-7-methyl-5*H*-thiazolo[3,2-α]pyrimidin-5-one, termed R59022 (*6*), and 3-(2-{4-[bis(4-fluorophenyl)methylene]-1-piperidinyl}ethyl)-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone, known as R59949 (*7*). R59949 appears to bind to the catalytic domain of the enzyme (*8*). Neither of these inhibitors is very specific for a particular isoform of DGK, although R59949 exhibited somewhat greater inhibition with calcium-dependent isoforms of this enzyme (*8*). With DGK isoforms from *Arabidopsis thaliana*, R59022 exhibits greater inhibition with AtDGK2 than with another isoform from this organism, AtDGK7 (*9*). These two inhibitors have been used extensively in pharmacological studies of the role of DGK in mammalian cells (see, for example, refs *10*−*14*).

One would anticipate that lipids having a close structural relationship with the natural substrates, 1,2-DAGs, would be either inhibitors or themselves substrates of DGK. The structurally related 1,3-DAGs are not substrates for DGKζ (*15*). Ceramide, a lipid with a single hydroxyl group like DAG, was found to be a competitive inhibitor of a Ca^{2+}-dependent isoform of DGK (*16*). It has also been shown recently that a homologue of the mammalian diacylglycerol kinases, YegS, has specificity for an unrelated lipid, phosphatidylglycerol (*17*).

Enantiomers (mirror-image forms) of substrates are often found to be inhibitors of enzymes. In the case of the substrate

† This work was supported by grants from the Canadian Natural Sciences and Engineering Research Council (Grant 9848) and the National Institutes of Health (Grant CA95463).

* To whom correspondence should be addressed. Telephone: (905) 525-9140, ext. 22073. Fax: (905) 521-1397. E-mail: epand@mcmaster.ca.

‡ McMaster University.

§ University of California.

|| University of Washington.

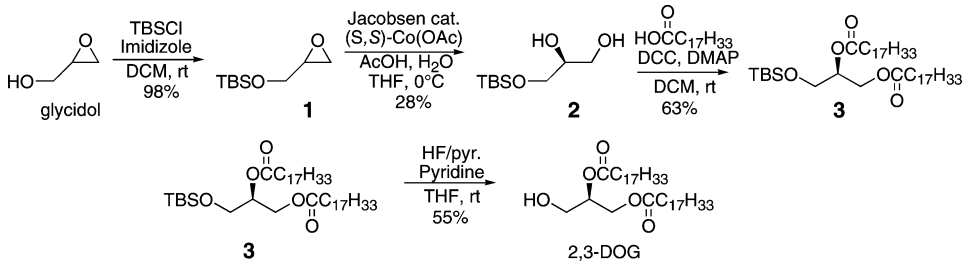
⊥ National Research Council of Canada.

@ University of Utah.

[1] Abbreviations: DGK, diacylglycerol kinase; MuLK, multisubstrate lipid kinase; 2,3-DOG, 2,3-dioleoylglycerol; 1,2-DOG, 1,2-dioleoylglycerol; SAG, 1-stearoyl-2-arachidonoylglycerol; PA, phosphatidic acid; MOI, multiplicity of infection; CL, cardiolipin; DAG, diacylglycerol; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMAP, dimethylaminopyridine; DCM, dichloromethane.

10.1021/bi701584v CCC: $37.00
Published on Web 11/16/2007

Scheme 1: Revised Synthesis of 2,3-DOG



1,2-diacylglycerol, there is one chiral center at the C-2 position of glycerol. The enantiomer of 1,2-diacylglycerol is 2,3-diacylglycerol. We determined the chiral specificity of several forms of DGK as well as the action of 2,3-diacylglycerol as an inhibitor of MuLK and different isoforms of mammalian DGK.

EXPERIMENTAL PROCEDURES

Materials

Lipids, except for 2,3-dioleoylglycerol (2,3-DOG), 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 Perkin-Elmer (Boston, MA). Other chemicals were of the purest available grade. Double-distilled water was used for all solutions.

Synthesis of 2,3-DOG

An initial synthesis of 2,3-DOG was adapted from a general synthesis of diacylglycerols (*18*). This route provided initial samples for enzyme analysis; however, the purity of the products was not optimal, and a second, improved synthesis of 2,3-DOG was developed. The second route began with racemic glycidol. Glycidol was distilled and then reacted with *tert*-butyldimethylsilyl chloride (TBSCl) to form the TBS-protected glycidol **1** (Scheme 1). Epoxide **1** was then reacted with the Jacobsen's chiral (*S*,*S*)-salen cobalt catalyst under hydrolytic kinetic resolution conditions to yield diol **2** (*19*). This species was then acylated with oleic acid, using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), to yield 2,3-diacyl TBS ether **3**. After multiple deprotection methods had been screened, HF-pyridine conditions were found to be the most dependable. TBS ether **3** was reacted with HF-pyridine in excess pyridine to yield 2,3-DOG.

*TBS-Protected Glycidol (**1**)*. Glycidol was distilled via short path distillation (62 °C at 15 Torr). A mixture of 10 mL (0.15 mol) of glycidol, 22.61 g (0.150 mol) of TBSCl, and 12.25 g (0.18 mol) of imidazole in 500 mL of dichloromethane (DCM) was stirred at 0 °C for 1.5 h. The mixture was washed with NH_4Cl and brine and extracted with DCM, dried over sodium sulfate (overnight), filtered, and concentrated to yield 27.9 g (98%) of TBS-protected glycidol **1**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.10 (6 H, t, TBS methyls), 0.90 (9 H, m, TBS tBu); glycidol backbone protons δ 2.60 (1 H, dd), 2.75 (1 H, dd), 3.10 (1 H, m, methine), 3.70 (1 H, dd), 3.85 (1 H, dd).

*2,3-Diol TBS Ether (**2**)*. A sample of 160.4 mg (2.65 mmol) of Jacobsen (*S*,*S*)-Co(salen)(OAc) catalyst was dissolved in 4 mL of toluene, to which 60 μL (1.06 mmol) of AcOH was added (*19*). This mixture was stirred open to air, and toluene was then removed under reduced pressure. A sample of 10 g (53.1 mmol) of epoxide **1** was added to this activated catalyst species along with 1 mL of THF. The solution was cooled to 0 °C, and 430 μL of H_2O (23.9 mmol) was added dropwise. The mixture was stirred overnight. The diol product was separated from the mixture by fractional distillation to yield of 3.11 g (14.9 mmol, 28%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.10 (6 H, t, TBS methyls), 0.90 (9 H, m, TBS tBu), 1.65 (1 H, s, OH), 2.60 (1 H, s, OH), 2.70 (6 H, m, carbon backbone and trace DCM).

*2,3-Diacyl TBS Ether (**3**)*. A sample of 504 mg (2.44 mmol) of diol **2** was dissolved in 10 mL of dry DCM. To this solution were added 1.52 g (5.37 mmol) of oleic acid, 656 mg (5.37 mmol) of DMAP, and 2.27 g (11.0 mmol) of DCC. After being stirred for 48 h at 23 °C, the mixture was extracted with DCM, dried over sodium sulfate, filtered, and concentrated. Purification of this mixture was very difficult, and multiple chromatographies over silica gel were run with different solvent gradients (0 to 2% EtOAc/Hex). Following this process, 1.12 g (63%) of pure diacyl lipid **3** was isolated: ^{1}H NMR (500 MHz, $CDCl_3$), δ 0.10 (6 H, t, TBS methyls), 0.90 (9 H, m, TBS tBu), 3.70 (2 H, d, acylO-CH_2), 4.20 (1 H, dd, TBSO-CH_2), 4.35 (1 H, dd, TBSO-CH_2), 5.10 (1 H, m, methine), 5.35 (4 H, m, CH═CH).

2,3-DOG. A sample of 200 mg (27.2 mmol) of diacyl TBS ether **3** was added to a small Nalgene bottle along with 3 mL of THF and 0.5 mL of pyridine. A few drops of HF-pyridine was added, and the mixture was stirred overnight. The mixture was suspended in sodium bicarbonate and extracted with ether, dried over sodium sulfate, filtered, and concentrated. The mixture was purified on a small column (10% EtOAc/Hex) to yield 94 mg (15 mmol, 55%) of 2,3-DOG: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (6 H, t, CH_3-), 1.30 (~42 H, d, hydrocarbon), 1.50 (4 H, m, hydrocarbon), 2.05 (8 H, m, hydrocarbon), 2.35 (4 H, m, -O-CH_2-), 3.75 (2 H, d, acylO-CH_2), 4.25 (1 H, dd, HO-CH_2), 4.35 (1 H, dd, HO-CH_2), 5.10 (1 H, m, methine), 5.35 (4 H, m, CH═CH).

While many enantiomeric purity analyses were explored, Mosher's ester derivatization proved to be the most useful. Precedent for this method from the related 2,3-DPG analysis had been developed (*18*). Many physical separation methods for determination of the ee (enantiomeric excess, i.e., the difference in amount between one enantiomer and the other) of the 2,3-DOG sample (chiral HPLC, chiral GC) failed to separate the enantiomers of the diacylglycerol. The Mosher's ester synthesis used a procedure involving the formation of Mosher's chloride in situ, followed by a direct treatment of the acyl chloride with the chiral alcohol under investigation (*20*).

Synthesis of Mosher's Acid Chloride. Approximately 10 mg of (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) was treated with excess (>5 equiv) oxalyl chloride and a slight excess (>2 equiv) of DMF in 1 mL of hexanes. This mixture was stirred for 1 h. The precipitate of the reaction was then removed by filtration through a cotton plug, and the hexanes containing MTPACl were added to a flask containing the 2,3-DOG sample and pyridine.

Mosher's Ester Derivatization. A vial was prepared containing 4.9 mg (7.80 μmol) of the 2,3-DOG sample and an excess of pyridine. The MTPACl/hexane solution was filtered into the vial, and a small crystal of DMAP was added. The mixture was allowed to stand overnight. Crude NMR analysis showed complete conversion of the 2,3-DOG sample to a Mosher's derivative. The presence of two doublets of doublets in the region of 4.2−4.4 ppm (and absence of two) indicated an enantiomeric ratio of greater than 95:5. A racemic standard was synthesized to compare with the 2,3-DOG spectra. The racemate showed the presence of four doublets of doublets in the region of 4.2−4.4 ppm, agreeing with the literature report (*18*), and supporting the enantiomeric analysis of the 2,3-DOG sample. It was concluded on the basis of the established data from the Jacobsen transformation and the observations of the Mosher's derivatization that 2,3-DOG was synthesized at an enantiomeric ratio of at least 95:5.

Preparation of DGKζ and DGKε

cDNAs encoding human DGKζ with a C-terminal FLAG epitope tag or human DGKε with a C-terminal six-His tag were cloned into BacPAK6 (Clontech), and baculovirus stocks were generated using the BacPAK system protocol (Clontech). Following the BacPAK system protocol, Sf21 cells were cultured and then infected with the virus stocks. Seventy-two hours later, cells were harvested in 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 1 mM DTT, 1 mM PMSF, 0.5 mM sodium orthovanadate, and leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor (10 μg/mL each). The pellet was collected by centrifugation at 6000*g* for 5 min at 4 °C and stored at −80 °C until it was used. Expression of recombinant protein was verified by Western blot analysis using anti-DGKζ, anti-DGKε, or anti-His antibodies (Penta-his, Qiagen Inc., Mississauga, ON).

Preparation of DGKα

cDNA encoding human DGKα was cloned into pBlue-BacHis (Invitrogen), which attaches an N-terminal six-His tag. As described above, baculovirus stocks were prepared and used to infect Sf-9 insect cells, which were harvested 50 h post-infection in the buffer described for DGKζ and -ε. The Sf-9 cells were routinely maintained in SF 900 II serum free medium (Invitrogen Life Technologies, Carlsbad, CA) in Erlenmeyer flasks (Corning; 125−250 mL, working volume of 30−60 mL) agitated in an incubator shaker at 110 rpm and 27 °C. Production of recombinant protein was conducted in Erlenmeyer shaker flasks (1 L, working volume of 500 mL) by infecting cells growing exponentially at a density of $2.5-3 \times 10^6$ cells/mL with the baculovirus at a MOI of 10 plaque forming units per cell. The infection process was monitored by measuring the cell number and the increase in average cell size. The culture was harvested 50 h post-infection, at a final viability of 90%. The culture supernatant was centrifuged at 500*g* (J6B, Beckman, Fullerton, CA) for 10 min at 4 °C. The cell pellets were collected and washed once with PBS and then stored −80 °C until they were used. The production of recombinant protein was verified by Western blot analysis using anti-His antibody (Penta-his, Qiagen Inc.).

Purification of Recombinant MuLK-FLAG from Escherichia coli

A protease deficient strain of *E. coli*, BL-21, was used for expression of this protein. MuLK-FLAG fusion protein was expressed in the pFLAG-CTC vector and purified with the FLAG affinity gel as follows. Briefly, after induction with 100 μM IPTG for 4 h at 30 °C, cells expressing either a control fusion protein (Synaptic Vesicle Protein 2, amino truncation) or MuLK-FLAG were harvested, sonicated, and centrifuged for 20 min at 20000*g*. Lysates were incubated for 16 h with FLAG-affinity gel (Sigma) and subsequently washed in a batchwise manner three times with 10 mM MOPS (pH 7.2), 25 mM NaCl, and 1 mM EGTA (wash buffer), one time with 10 mM MOPS, 300 mM NaCl, and 1 mM EGTA, and one additional time with wash buffer. The FLAG-tagged protein was then eluted with 100 μg/mL 3× FLAG peptide (Sigma) in 10 mM MOPS (pH 7.2), 50 mM NaCl, 1 mM EGTA, and protease inhibitors (Sigma). The eluted purified protein was stored in 20% (v/v) ethylene glycol (Fisher) at −20 °C. The protein concentration of the eluted purified protein was determined by a BCA protein assay.

Assay of the Enzymatic Activity of DGKα, -ε, and -ζ

The Triton micelle-based assay was based on published procedures (*22*−*24*). Briefly, a cell pellet from cells overexpressing either DGKα or DGKζ was extracted with 2 M KCl in 33 mM Tris-HCl (pH 8.0) with 67 mM NaCl and 5 μg/mL histone. The cells were spun in an ultracentrifuge for 30 min at 100000*g* and 20 °C. In the case of DGKε, the enzyme was not efficiently extracted from the cell pellet with a salt solution but was solubilized by addition of 30 mM octyl glucoside to the KCl solution. The mixture was briefly vortexed and placed in a 30 °C water bath for 10 min.

A lipid film was made from a solution of lipid in $CHCl_3$ and CH_3OH (2:1) by evaporation of the solvent under a stream of N_2. The lipid was deposited on the walls of a glass culture test tube and then placed under vacuum for at least 2 h. The lipid film was solubilized in a Triton buffer, the enzyme preparation added, and the reaction mixture equilibrated for 5 min at 25 °C. The reaction was initiated by adding 20 μL of 5 mM [γ-^{32}P]ATP (50 μCi/mL). The final assay mixture contained 15 mM Triton X-100, 50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA (pH 7.2), 1 mM DTT, and enzyme either with or without 2.5 mM Ca^{2+}, as well as phospholipid and DAG as indicated. The reaction was carried out for 10 min at 25 °C and was terminated by the addition of 2 mL of a $CHCl_3$/CH_3OH mixture (1:1, v/v) containing 0.25 mg/L dihexadecyl phosphate. The organic phase was washed three times with 2 mL each of 1% $HClO_4$ and 0.1% H_3PO_4 in a H_2O/methanol mixture (7:1, v/v). The volume of the final organic phase was 0.80 mL. A 0.40 mL

Table 1: Chiral Specificities of DGK Isoforms

isoform	% activity[a]	conditions
DGKα	8.5 ± 1	0.8 mM DOG, 5 mM DOPS
DGKζ	12 ± 2	0.8 mM DOG, 5 mM DOPS
DGKϵ	6 ± 1[b]	1 mM DOG, 18 mM DOPC
MuLK	33 ± 1	2.5 mM DOG, 1 mM CL

[a] Rate of DGK-catalyzed phosphorylation of 2,3-DOG to 1,2-DOG expressed as a percentage. [b] The % activity of DGKϵ against 2,3-DOG vs the preferred substrate for this isoform, i.e., 1-stearoyl-2-arachidonoyl glycerol, is 2.3 ± 0.4.

aliquot of the organic phase was placed in a scintillation vial to assess the incorporation of ^{32}P into phosphatidic acid by Cerenkov counting. Without the addition of exogenous lipid substrate, there was no incorporation of ^{32}P from ATP into endogenous lipids. Blanks were run without the addition of enzyme or with enzyme preparations from mock transfected cells. The assay was carried out in triplicate, and the results are presented with errors showing the standard deviation of the mean for one experiment.

Assay of Enzymatic Activity of MuLK

A dried lipid film was prepared as described above containing a DAG and tetraoleoyl cardiolipin (CL). The lipid film was dissolved in Triton buffer to give final concentrations in the assay of 1 mM CL, a specified concentration of DAG, 50 mM Triton X-100, 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 10 mM MOPS (pH 7.2), a protease inhibitor cocktail for use with mammalian cell and tissue culture extracts [Sigma, catalog no. P 8340 at a 1:100 (v/v) dilution], 1 mM DTT, an amount of Ca^{2+} calculated to give 1 nM free calcium, and 3 μg of MuLK (or the control fusion protein, Synaptic Vesicle Protein 2, amino truncation) in 20% ethylene glycol. The mixture was briefly vortexed and placed in a 30 °C water bath for 10 min. The reaction was initiated by addition of 20 μL of 2.5 mM [γ-^{32}P]ATP (250 μCi/mL) to give a final volume of 100 μL. The reaction was generally terminated after 20 min and the phosphatidic acid product extracted and counted as described above.

RESULTS

The activities of several enzymes that phosphorylate DAG were compared in terms of their relative activity against 1,2-DOG versus 2,3-DOG (Table 1). The rate of phosphorylation of 1,2-DAG was found to be linear over 10 min for the DGKs and 20 min for MuLK. The negative control for MuLK, Synaptic Vesicle Protein 2, exhibited less than 10% of the activity of MuLK. In all cases, 1,2-DOG is a better substrate than its enantiomer. However, the relative ability of MuLK to phosphorylate 2,3-DOG is much greater than that for any of the isoforms of DGK used (Table 1). In the case of the DGK isoforms, some of the activity against 2,3-DOG could be a consequence of the presence of ~5% 1,2-DOG as an enantiomeric impurity. The relatively higher activity of MuLK against 2,3-DOG compared with the DGKs allowed us to determine the kinetic constants for this enzyme (Figure 1). The measured rates of this enzyme against 1,2-DOG are lower than those previously reported (*3*); however, the conditions of the assay are not identical, and the enzyme preparation we used had been frozen, stored for several days in this state, and then thawed for use, likely contributing to the somewhat lower activity observed in this work. The V_{max} and K_m values for 2,3-DOG are 10.21 ± 0.04 nmol min^{-1} mg^{-1} and 0.379 ± 0.002 mol %, respectively, compared with 23.6 ± 0.5 nmol min^{-1} mg^{-1} and 0.309 ± 0.009 mol % for 1,2-DOG, respectively. Thus, at V_{max} the activity of MuLK against 1,2-DOG is only ~2-fold greater than against 2,3-DOG.

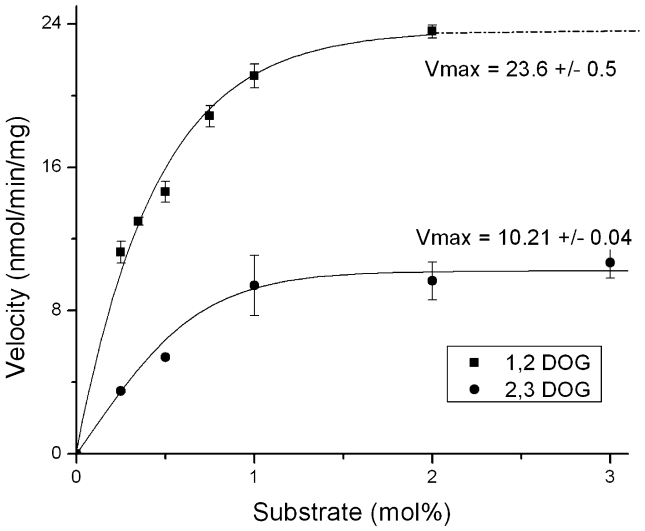


FIGURE 1: Substrate concentration dependence of MuLK-catalyzed phosphorylation of 1,2-DOG and 2,3-DOG: (■) 1,2-DOG and (●) 2,3-DOG. The data are fitted to the Michaelis−Menten equation giving values for the constants V_{max} and K_m for 1,2-DOG of 23.6 ± 0.5 nmol min^{-1} mg^{-1} and 0.309 ± 0.009 mol %, respectively. The V_{max} and K_m for 2,3-DOG are 10.21 ± 0.04 nmol min^{-1} mg^{-1} and 0.379 ± 0.002 mol %, respectively. The data represented are averages of two trials of the experiment.

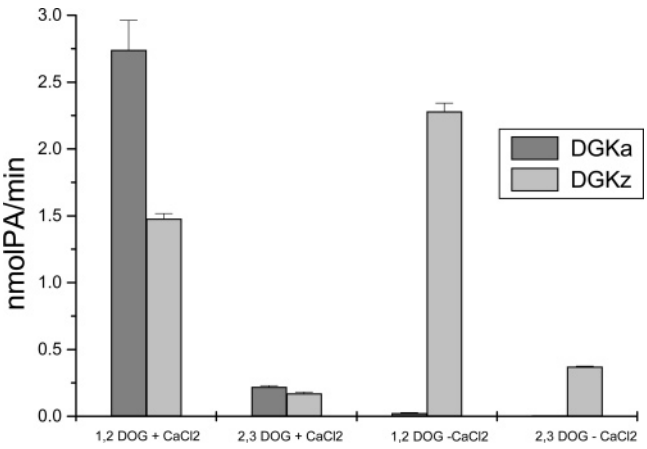


FIGURE 2: Comparison of the activities of DGKα and DGKζ on 1,2-DOG and 2,3-DOG in the presence and absence of 2.5 mM $CaCl_2$: DGKα (darker bars) and DGKζ (lighter bars). Assay conducted with 1 mM EGTA and 5 mM Mg^{2+} at pH 7.2 and 25 °C. Other conditions as described in Experimental Procedures.

For both DGKα and DGKζ, there was a much greater activity against 1,2-DOG than 2,3-DOG, both in the presence and in the absence of Ca^{2+} (Figure 2). DGKα is a calcium-dependent enzyme (*25*) and consequently has much higher activity in the presence of this cation. Ca^{2+} causes a small inhibition of the activity of DGKζ. This is likely a nonspecific effect caused by the high Ca^{2+} concentration of 2.5 mM that was used. MuLK was assayed in the presence of CL, rather than with the lipids used for the DGK assays. For all of the lipid kinases used in this work, the assay conditions

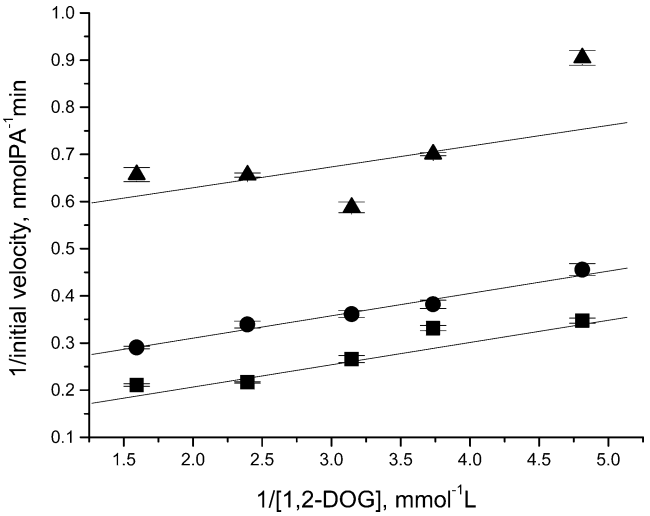


FIGURE 3: Lineweaver−Burk plot for DGKα activity phosphorylation of 1,2-DOG in the presence of 2.5 mM Ca^{2+} and 0 (■), 0.42 (●), or 0.84 mM 2,3-DOG (▲).

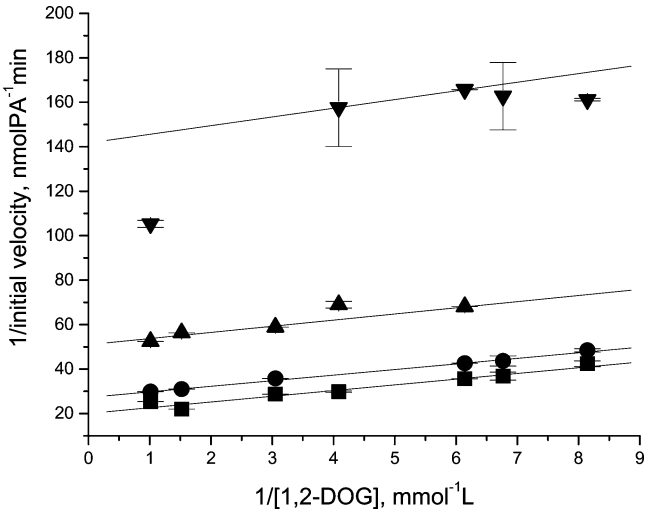


FIGURE 4: Lineweaver−Burk plot for DGKζ activity phosphorylation of 1,2-DOG in the presence of 0 (■), 0.165 (●), 0.33 (▲), or 0.66 mM 2,3-DOG (▼). Data were combined from six independent experiments and normalized relative to the initial velocity observed in the absence of 2,3-DOG.

were generally chosen to produce high enzymatic activity. One would not anticipate the assay conditions would substantially affect the chiral specificity of the enzymes, as we illustrate in Figure 2 for the presence and absence of Ca^{2+}. The Michaelis−Menten constants for the phosphorylation of 2,3-DOG by either DGKα or DGKζ were not determined because of the low activity observed with this substrate and because some of this activity likely originates from the presence of 1,2-DOG impurities in the 2,3-DOG preparation. However, we did observe that adding a 5- or 10-fold excess of 2,3-DOG over the concentration of 1,2-DOG resulted in a significant inhibition of the rate of production of phosphatidic acid both in the presence and in the absence of Ca^{2+}. DGKα was assayed in the presence of 2.5 mM Ca^{2+} and with 0, 0.42, or 0.84 mM 2,3-DOG as a function of 1,2-DOG concentration. Plotting the results as a Lineweaver−Burke plot (Figure 3) indicates that the inhibition of the phosphorylation of 1,2-DOG by 2,3-DOG is characteristic of being uncompetitive. Note that since these are double-reciprocal plots, the spacings of the lines are not proportional to the concentration of inhibitor used. In uncompetitive inhibition, both V_{max} and K_m are affected, in contrast to competitive inhibition in which only K_m is altered or to noncompetitive inhibition in which only V_{max} is changed. A similar analysis of the inhibition of DGKζ phosphorylation of 1,2-DOG by 2,3-DOG also indicates that the inhibition is uncompetitive (Figure 4). The kinetic data were further analyzed to obtain the kinetic constants for the phosphorylation of 1,2-DOG by DGKα and DGKζ and its inhibition by 2,3-DOG (Table 2).

Unlike DGKα or DGKζ, the ϵ isoform of DGK does not exhibit competitive inhibition between 2,3-DOG and either 1,2-DOG or SAG (1-stearoyl-2-arachidonoylglycerol) (Figure 5). The activity of DGKϵ is greater with SAG than with 1,2-DOG as expected due to the arachidonoyl specificity of this isoform. However, addition of 2,3-DOG to either of these two substrates results in increased activity (Figure 5), rather than inhibition as seen with DGKα and DGKζ. This is likely a result, at least in part, of 2,3-DOG being a weak substrate for DGKϵ (Table 1). Thus, DGKϵ differs from DGKα and DGKζ in that 2,3-DOG does not inhibit the enzyme.

Table 2: Kinetic Constants for Phosphorylation of 1,2-DOG by DGKα and DGKζ and Inhibition by 2,3-DOG

	1,2-DOG		2,3-DOG
enzyme	K_m (mol %)	V_{max} (nmol of PA/min)[a]	K_I (mol %)
DGKα	0.8 ± 0.3	5.5 ± 0.4	1.7 ± 0.2
DGKζ	0.52 ± 0.08	0.050 ± 0.002	1.07 ± 0.07

[a] The value of V_{max} is a measure of the maximal rate for the amount of enzyme used, but it is not normalized for the concentration of enzyme, which is not known.

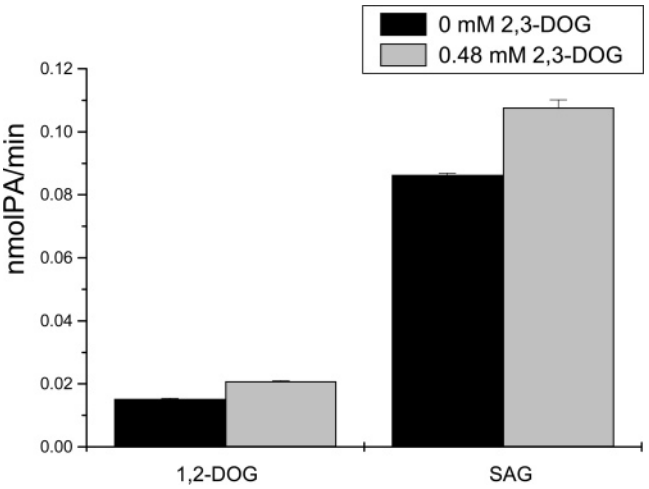


FIGURE 5: Comparison of DGKϵ activity on 1,2-DOG and SAG in the absence (black bars) and presence of 0.48 mM 2,3-DOG (gray bars). Assay conducted with 0.24 mM 1,2-DOG or SAG and 75 mM OG instead of Triton X-100. Other conditions as described in Experimental Procedures.

DISCUSSION

All three DGK isoforms used in this work exhibited a high degree of chiral specificity for the substrate (Table 1). It had also been observed that protein kinase C was much less activated by 2,3-DAG than by 1,2-DAG, although the binding affinity of 2,3-DAG for protein kinase C was only 4-fold lower than for 1,2-DAG (*26*). MuLK also phosphorylated 1,2-DOG more rapidly than 2,3-DOG, but with chiral

specificity several-fold lower compared with that of the DGKs.

The chiral specificity of MuLK is not as great as that of the DGKs. Its greater activity with 2,3-DOG may reflect a relationship to prokaryotic lipid kinases that are less specific with regard to the substrate they phosphorylate. As with bacterial lipid kinases, MuLK can also phosphorylate ceramide (*3*), although ceramides are not substrates for mammalian DGKs. MuLK phosphorylates several different lipid substrates and for that reason has been termed a multiple lipid kinase. MuLK has low substrate specificity and particularly low chiral specificity since both 1,2-DOG and 2,3-DOG are phosphorylated. However, there is partial chiral specificity, since 1,2-DOG is preferred as substrate. Interestingly, archaebacteria have opposite lipid chiralities compared with those from other species, having their major phospholipids as derivatives of 2,3-DAG (*27*).

With the DGKs, 2,3-DOG is an inhibitor of the phosphorylation of 1,2-DOG, although it is not a potent inhibitor, requiring a concentration of 2,3-DOG on the order of 5−10-fold higher to cause 50% inhibition of the phosphorylation of 1,2-DOG, depending on the specific isoform and conditions. Analysis of the kinetics indicates that the inhibition is uncompetitive, as indicated by the fact that the Lineweaver−Burke plots with and without the 2,3-DOG inhibitor are parallel to each other for both DGKα (Figure 3) and DGKζ (Figure 4). Uncompetitive inhibition occurs when the inhibitor binds to the enzyme−substrate complex, but not to the free enzyme. This differs from both competitive inhibition in which the inhibitor binds only to the free enzyme and noncompetitive inhibition in which the inhibitor can bind equally well to the free enzyme and the enzyme−substrate complex. Uncompetitive inhibition is not the type of inhibition one would expect for the enantiomer of a substrate that would *a priori* be anticipated to compete with the substrate for the active site and hence exhibit competitive inhibition kinetics. The results suggest that the binding of 1,2-DOG to the active site induces a conformational change that exposes a site on the enzyme that can bind 2,3-DOG. The DGK isoforms that we used each have two C1 domains. C1 domains are known to generally bind to DAG. However, the C1 domains of DGKs that we tested have not been shown to bind either DAG or phorbol esters in vitro. It has been shown that the C1 domains of DGKα are not involved in the binding of DAG to the active site (*28*). We suggest that the active site of DGK binds DAG with chiral specificity; i.e., it binds only 1,2-DAG and not 2,3-DAG. This process does not directly involve the C1 domains. However, we suggest that the binding of 1,2-DAG to the active site induces a conformational change in the enzyme that makes the C1 domains more accessible to binding DAG in a nonstereospecific manner; i.e., the enzyme can now bind either 1,2-DAG or 2,3-DAG, albeit with lower affinity. This phenomenon results in the uncompetitive inhibition by 2,3-DAG and also causes deviations from Michaelis−Menten kinetics at high concentrations of 1,2-DAG, resulting in a lowering of the maximal rate at high substrate concentrations (unpublished results).

There is an interesting gradation of chiral specificity among the enzymes that were tested. It is known that the chiral specificity of interfacial catalysis is dependent on the structure of the interface (*29*−*31*). With the group of enzymes used in this study, there is a relationship between the substrate specificity and the chiral specificity. Thus, MuLK, which can phosphorylate ceramide as well as DAG, is the least specific enzyme and also the one with the highest activity against 2,3-DOG, showing that it also has low chiral specificity. DGKε is the most specific among the enzymes tested since it not only is specific for 1,2-diacylglycerol but also discriminates among 1,2-diacylglycerols, depending on the nature of the acyl chain. This is the only DGK isoform tested in which 2,3-DOG is neither a good substrate nor an inhibitor. DGKα and DGKζ are intermediate cases in which 2,3-DOG is a poor substrate, but it can act as an inhibitor. This gradation of properties is not related to the binding affinities of the lipids for these enzymes, which do not differ very much. It is dependent on the specificity, rather than the affinity, of binding.

We have thus shown that DGKα, -ζ, and -ε exhibit a high degree of stereospecificity in catalyzing the phosphorylation of DAG. This would result in the stereospecific formation of PA, which could be further converted to other phospholipid components of the cell membrane that would also be 1,2-diacyl lipids. The stereospecificity of MuLK is much lower, in accord with its designation as a multiple lipid kinase.

ACKNOWLEDGMENT

We thank Barbara Jardin and Rosa Tran for their assistance in the production of DGKα.

REFERENCES

1. Topham, M. K. (2006) Signaling roles of diacylglycerol kinases, *J. Cell. Biochem. 97*, 474−484.
2. Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007) Diacylglycerol kinases: Why so many of them? *Biochim. Biophys. Acta 1771*, 793−806.
3. Waggoner, D. W., Johnson, L. B., Mann, P. C., Morris, V., Guastella, J., and Bajjalieh, S. M. (2004) MuLK, a eukaryotic multi-substrate lipid kinase, *J. Biol. Chem. 279*, 38228−38235.
4. Bektas, M., Payne, S. G., Liu, H., Goparaju, S., Milstien, S., and Spiegel, S. (2005) A novel acylglycerol kinase that produces lysophosphatidic acid modulates cross talk with EGFR in prostate cancer cells, *J. Cell Biol. 169*, 801−811.
5. Gorzelle, B. M., Nagy, J. K., Oxenoid, K., Lonzer, W. L., Cafiso, D. S., and Sanders, C. R. (1999) Reconstitutive refolding of diacylglycerol kinase, an integral membrane protein, *Biochemistry 38*, 16373−16382.
6. de Chaffoy de Courcelles, D., Roevens, P., and Van Belle, H. (1985) R 59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet, *J. Biol. Chem. 260*, 15762−15770.
7. de Chaffoy de Courcelles, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y., and De Clerck, F. (1989) The role of endogenously formed diacylglycerol in the propagation and termination of platelet activation. A biochemical and functional analysis using the novel diacylglycerol kinase inhibitor, R 59 949, *J. Biol. Chem. 264*, 3274−3285.
8. Jiang, Y., Sakane, F., Kanoh, H., and Walsh, J. P. (2000) Selectivity of the diacylglycerol kinase inhibitor 3-[2-(4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl)ethyl]-2,3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) among diacylglycerol kinase subtypes, *Biochem. Pharmacol. 59*, 763−772.
9. Gómez-Merino, F. C., Arana-Ceballos, F. A., Trejo-Téllez, L. I., Skirycz, A., Brearley, C. A., Dörmann, P., and Mueller-Roeber, B. (2005) *Arabidopsis* AtDGK7, the smallest member of plant diacylglycerol kinases (DGKs), displays unique biochemical features and saturates at low substrate concentration: The DGK inhibitor R59022 differentially affects AtDGK2 and AtDGK7 activity *in vitro* and alters plant growth and development, *J. Biol. Chem. 280*, 34888−34899.
10. Cooke, E., Al-Mohanna, F. A., and Hallett, M. B. (1987) Diacylglycerol kinase inhibitor, R59022, potentiates neutrophil

oxidase activation by Ca^{2+}-dependent stimuli. Evidence for two separate but convergent pathways, *Biochem. Pharmacol. 36*, 3459−3462.
11. Gomez-Cambronero, J., Molski, T. F., Becker, E. L., and Sha'afi, R. I. (1987) The diacylglycerol kinase inhibitor R59022 potentiates superoxide production but not secretion induced by fMet-Leu-Phe: Effects of leupeptin and the protein kinase C inhibitor H-7, *Biochem. Biophys. Res. Commun. 148*, 38−46.
12. Alonso, R., Rodríguez, M. C., Pindado, J., Merino, E., Mérida, I., and Izquierdo, M. (2005) Diacylglycerol kinase α regulates the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes, *J. Biol. Chem. 280*, 28439−28450.
13. Alonso, R., Mazzeo, C., Merida, I., and Izquierdo, M. (2007) A new role of diacylglycerol kinase α on the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes, *Biochimie 89*, 213−221.
14. Batista, E. L., Jr., Warbington, M., Badwey, J. A., and Van Dyke, T. E. (2005) Differentiation of HL-60 cells to granulocytes involves regulation of select diacylglycerol kinases (DGKs), *J. Cell. Biochem. 94*, 774−793.
15. Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996) Molecular cloning and characterization of a novel human diacylglycerol kinase ζ, *J. Biol. Chem. 271*, 10230−10236.
16. Younes, A., Kahn, D. W., Besterman, J. M., Bittman, R., Byun, H. S., and Kolesnick, R. N. (1992) Ceramide is a competitive inhibitor of diacylglycerol kinase in vitro and in intact human leukemia (HL-60) cells, *J. Biol. Chem. 267*, 842−847.
17. Bakali, H. M. A., Herman, M. D., Johnson, K. A., Kelly, A. A., Wieslander, A., Hallberg, B. M., and Nordlund, P. (2007) Crystal Structure of YegS, a Homologue to the Mammalian Diacylglycerol Kinases, Reveals a Novel Regulatory Metal Binding Site, *J. Biol. Chem. 282*, 19644−19652.
18. Vilcheze, C., and Bittman, R. (1994) An efficient asymmetric synthesis of diacylglycerols, *J. Lipid Res. 35*, 734−738.
19. Schaus, S. E., Brandes, B. D., Larrow, J. F., Tokunaga, M., Hansen, K. B., Gould, A. E., Furrow, M. E., and Jacobsen, E. N. (2002) Highly selective hydrolytic kinetic resolution of terminal epoxides catalyzed by chiral (salen)Co(III) complexes. Practical synthesis of enantioenriched terminal epoxides and 1,2-diols, *J. Am. Chem. Soc. 124*, 1307−1315.
20. Ward, D. E., and Rhee, C. K. (1991) A Simple Method for the Microscale Preparation of Mosher Acid Chloride, *Tetrahedron Lett. 32*, 7165−7166.
21. Tom, R. L., Caron, A. W., Massie, B., and Kamen, A. A. (1995) Scale-up of recombinant virus and protein production in stirred-tank reactors, *Methods Mol. Biol. 39*, 203−224.
22. 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.
23. 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.
24. Epand, R. M., Kam, A., Bridgelal, N., Saiga, A., and Topham, M. K. (2004) The α isoform of diacylglycerol kinase exhibits arachidonoyl specificity with alkylacylglycerol, *Biochemistry 43*, 14778−14783.
25. 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.
26. Johnson, J. E., Zimmerman, M. L., Daleke, D. L., and Newton, A. C. (1998) Lipid structure and not membrane structure is the major determinant in the regulation of protein kinase C by phosphatidylserine, *Biochemistry 37*, 12020−12025.
27. Kates, M., Moldoveanu, N., and Stewart, L. C. (1993) On the revised structure of the major phospholipid of *Halobacterium salinarium*, *Biochim. Biophys. Acta 1169*, 46−53.
28. Sakane, F., Kai, M., Wada, I., Imai, S., and Kanoh, H. (1996) The C-terminal part of diacylglycerol kinase α lacking zinc fingers serves as a catalytic domain, *Biochem. J. 318* (Part 2), 583−590.
29. Fernandez-Lorente, G., Fernandez-Lafuente, R., Palomo, J. M., Mateo, C., Bastida, A., Coca, J., Haramboure, T., Hernandez-Justiz, O., Terreni, M., and Guisan, J. M. (2001) Biocatalyst engineering exerts a dramatic effect on selectivity of hydrolysis catalyzed by immobilized lipases in aqueous medium, *J. Mol. Catal. B: Enzym. 11*, 649−656.
30. Sabbani, S., Hedenstrom, E., and Nordin, O. (2006) The enantioselectivity of *Candida rugosa* lipase is influenced by the particle size of the immobilising support material Accurel, *J. Mol. Catal. B: Enzym. 42*, 1−9.
31. Palomo, J. M., Segura, R. L., Fernandez-Lorente, G., Fernandez-Lafuente, R., and Guisan, J. M. (2007) Glutaraldehyde modification of lipases adsorbed on aminated supports: A simple way to improve their behaviour as enantioselective biocatalyst, *Enzyme Microb. Technol. 40*, 704−707.

BI701584V