Lipid Modulation of the Activity of Diacylglycerol Kinase α- and ζ-Isoforms: Activation by Phosphatidylethanolamine and Cholesterol†

Maria Laura Fanani,‡ Matthew K. Topham,§ James P. Walsh,|| and Richard M. Epand*,‡

Department of Biochemistry and Biomedical Sciences, McMaster University Health Science Center, Hamilton, Ontario L8N 3Z5, Canada, Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84112, and Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Received April 28, 2004; Revised Manuscript Received September 2, 2004

ABSTRACT: Diacylglycerol kinase (DGK) isoforms α and ζ were extracted from transfected cells that overexpressed these enzymes. We determined the lipid dependence of the binding of these isoforms to liposomes. The modulation by lipid of the rate of phosphorylation of diacylglycerol by these enzymes was also measured. Incorporation of phosphatidylethanolamine into the liposomes resulted in an increased partitioning of both isoforms of DGK to the membrane as well as an increased catalytic rate. We demonstrate that the increased catalytic rate is a consequence of both increased portioning of the enzyme to the membrane and increased catalytic activity of the membrane-bound form. DGKα, a calcium-dependent isoform, can be activated in a calcium-independent fashion in the presence of phosphatidylethanolamine. Similar effects are observed with cholesterol. In contrast, sphingomyelin inhibits the activity of both isoforms of DGK. Our results demonstrate that the translocation to membranes and activity of DGKα and DGKζ are modulated by the composition and properties of the membrane. The enzymes are activated by the presence of lipids that promote the formation of inverted phases. However, the promotion of negative curvature is not the sole factor contributing to the lipid effects on enzyme binding and activity. A truncated form of DGKα lacking both the E-F hand and the recoverin homology domain is constitutively active and is not further activated by any of the lipids tested or by calcium. However, a truncated form lacking only the recoverin homology domain is partially activated by either calcium or certain lipids.

Mammalian diacylglycerol kinases (DGK)[1] have an important role in signal transduction. This enzyme catalyzes the removal of the important lipid messenger diacylglycerol from the cell membrane to produce another lipid messenger, phosphatidic acid (PA). The concentration of both lipid messengers in the membrane is closely regulated by DGK activity. We have studied two of the isoforms of DGK, α and ζ, that are members of type I and type IV DGKs, respectively (*1*, *2*). DGKα is found in the nucleus and cytosol of resting T-cells but is induced to translocate to the perinuclear region in response to IL-2 (*3*). DGKα will also translocate to the plasma membrane in response to activation of the T-cell antigen receptor (*4*). The DGKζ isoform is found in both the cytosol and the nucleus (*5*, *6*). The nucleus is also a site, independent of the plasma membrane, for phosphoinositide signaling, including the generation of diacylglycerol (*7*). There is also evidence that DGKζ associates with phosphatidylinositol 4-phosphate 5-kinase that leads to the polymerization of actin (*8*).

We investigate the interaction of DGKα and DGKζ with liposomes and the modulation of the activity and membrane binding of these enzymes by changes in the lipid environment. These enzymes have generally been studied using detergent mixed micelles, which have properties very different from cell membranes. In this work we use large unilamellar vesicles (LUVs), which more closely resemble cell membranes. In the present work we find that the sensitivity of the activity of DGK to changes in lipid composition is much greater with liposomes than had been found in a detergent micelle based assay (*9*). We are particularly interested in determining how the lipid environment of the membrane modulates the activity and membrane translocation of these isoforms. The activity of several enzymes that act at the membrane interface is known to be modulated by the physical properties of the membrane (*10*).

DGKα contains a calcium binding E-F hand motif, and the activity of this isoform is calcium dependent (*11*). However, it has been shown that when the enzyme is truncated by mutation and the E-F hand removed, it becomes constitutively active, independent of the presence of calcium (*12*, *13*). DGKα is also activated in a calcium-independent fashion by sonicated dispersions of several lipids (*14*).

† This work was supported by a grant from the Canadian Natural Sciences and Engineering Research Council (Grant 9848). M.L.F. is a postdoctoral fellow of Fundacion Antorchas, Argentina.

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

‡ McMaster University Health Science Center.

§ University of Utah.

|| Indiana University School of Medicine.

[1] Abbreviations: DGK, diacylglycerol kinase; LUV, large unilamellar vesicle; DOG, dioleoylglycerol; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DO, dioleoyl; DE, dielaidoyl; DPl, dipetroselinoyl; DTT, dithiothreitol; SLV, sucrose-loaded vesicles; TBS-T buffer, 50 mM Tris-HCl, 250 mM NaCl, and 0.1% Tween 20, pH 7.5; RVH, recoverin homology.

10.1021/bi049145z CCC: $27.50
Published on Web 10/27/2004

However, the significance of these findings is not clear since calcium is required for activity of this enzyme using other assay methods. No data have been reported for the assay of DGKα in which PS is a component of stable LUVs, and no lipid-specific activation has been shown using any assay system. Calcium and PS stimulate DGKα by different mechanisms (*12*). In comparison, DGKζ is not activated by calcium but is stimulated by phosphatidylserine (PS) (*9*). In the present work we demonstrate that the properties of membrane lipids strongly influence both the activity and binding of both isoforms of DGK to bilayer vesicles. In particular, DGKα can be activated in a calcium-independent fashion in the presence of certain lipids. This is analogous to the recent finding that phospholipids can switch the GTPase substrate preference of a GTPase-activating protein (*15*). It provides another example to illustrate the important influence that membrane lipids can have in modulating the activity of membrane-bound proteins even when they are not required as cofactors.

EXPERIMENTAL PROCEDURES

Materials. Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingomyelin was purified from eggs. 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 MP Biomedicals (Irvine, CA). Other chemicals were of the purest grade available. Doubly distilled water was used for all solutions.

Enzyme Preparations of DGKα and DGKζ. cDNA encoding a particular isoform was cloned into BacPAK6 (Clontech), and baculovirus stocks were generated using the BakPAK system (Clontech). Sf21 cells were infected with the virus stocks followed by harvesting of the cells 72 h later 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 10 μg/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. The protein concentration of the cell pellets was 19.5 ± 3 and 19.7 ± 2 mg/mL for the α- and ζ-isoforms, respectively. The enzyme was extracted from the cell pellets by first sonicating (5 min) followed by 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 were separated by centrifugation (100000*g*, 30 min, 20 °C). The 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.

Expression of Truncated Diacylglycerol Kinases in COS-1 Cells. DGKα-pCDNA3, DGKαΔ87-pCDNA3, and DGKαΔ196-pCDNA3, which express DGKα, DGKαΔ87, and DGKαΔ196, have been previously described (*13*). Each of these constructs includes a COOH-terminal FLAG epitope to facilitate quantification of expression by Western blotting. This modification does not alter DGK activity (*13*). COS-1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (Intergen), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Subconfluent cultures of COS-1 cells were transfected with truncated DGKα isoforms using Lipofectamine 2000 reagent (Invitrogen) as recommended by the supplier. Parallel control cultures were transfected with pCDNA3 vector only. After 48 h, cells were harvested and lysed by sonication in ice-cold 20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 100 mM NaCl, 1.0 mM EGTA, 1.0 mM $MgCl_2$, 1.0 mM DTT, 1 mM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM PMSF, and 50 μM ATP. After removal of undisrupted cells by brief centrifugation, the extracts were centrifuged at 100000*g* (Beckmann TL-100) for 20 min at 4 °C to pellet membranes. The supernatants were rapidly frozen in a dry ice/ethanol bath and stored at −70 °C until assayed.

Liposomal Assay of DGK Activity. 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 (50 mM Tris-HCl, 100 mM NaCl, pH 8.0). LUVs were made from this suspension by five freeze−thaw cycles and extruded 10 times through two 0.1 μm polycarbonate filters. The lipid composition of the LUVs was generally 5 mol % DOG, 40 mol % DOPS, and 55 mol % other lipids. For cases in which comparisons were made with liposomes containing both cholesterol and DOPE, the amount of DOPS was reduced to 30 mol % in order to have sufficient DOPC to maintain the liposomes in a bilayer phase.

DGK activity was assayed using a final concentration of 4.75 mM lipid as LUVs (or 12 mM in high lipid concentration experiments), 5 mM $MgCl_2$, 1 mM DTT, and 5 μL of 10× diluted, salt-extracted enzyme (7.5 μg of protein) with the addition of $CaCl_2$ and/or EGTA, as specified below. The final volume was 200 μL. 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_3OH (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 with the addition of mock-transfected cell lysates. 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 3 min.

Experiments at micromolar free calcium concentrations were carried out using calibrated EGTA−$CaCl_2$ buffers at pH 7.2. Free calcium concentrations were calculated using MaxChelator software and calibrated using Fura-2, a fluorescent probe sensitive to free calcium in the range 0.01−1.5 μM. Washing all the glassware with 5 mM EGTA solution and incubating the ATP for 1 h with the chelating resin, Chelex-100, avoided calcium contamination.

Membrane Binding Assay. A sucrose-loaded vesicle (SLV) binding assay was used. The SLVs were prepared by suspending lipid films in a buffer of 20 mM Tris-HCl containing 170 mM sucrose. The final lipid concentration of the suspension was 25 mM. This suspension was made into 100 nm diameter LUVs by extrusion, as described above. Five hundred microliters of these liposomes was diluted with 250 μL of assay buffer, followed by ultracentrifugation (30 min, 20 °C, 100000*g*). The top 500 μL was discarded, and the pellet was resuspended with 400 μL of assay buffer. Twenty-six microliters (39 μg of protein) of the salt-extracted DGK was added to 250 μL of washed SLV. Membrane-bound enzyme was separated from unbound enzyme by ultracentrifugation (30 min, 20 °C, 100000*g*). After centrifugation, the top 200 μL was diluted with 100 μL of assay buffer, and the bottom 100 μL was diluted with assay buffer to reach a final volume of 300 μL. The partitioning of DGK between the two fractions was determined by assaying the enzymatic activity using 71 μL of each fraction. The fraction of enzyme bound to the SLVs was determined as the percentage of the DGK activity of the bound fraction compared to the sum of DGK activities in the two fractions. In addition, the amount of DGKα in each of the two fractions was determined with Western blots using 10 μL of the fractions obtained after centrifugation using specific anti-DGK antibody. Scanning of Western blot bands was performed using the Scion Image program.

Other Methods. Total protein assay was performed using the Bio-Rad protein assay that is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. For immunodetection of DGK, Western blots were performed using approximately 1 μg of the enzyme sample (DGKα or the truncation mutants) applied to SDS−PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes (Ready gel blotting sandwich; Bio-Rad). After being blocked in TBS-T buffer (50 mM Tris-HCl, 250 mM NaCl, 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk for 1 h, the membrane was incubated with anti-DGKα or anti-FLAG antibody (1:2000 in TBS-T, 5% nonfat dry milk) for 1 h. Membranes were washed with TBS-T and incubated with peroxidase-conjugated secondary anti-IgG (1:2000 dilution in TBS-T, 5% nonfat dry milk) for 1 h. After being washed four times in TBS-T buffer, the peroxidase conjugates were detected by chemiluminescence. The assays were done in triplicate and confirmed in at least two independent experiments, and the error was expressed as the standard error of the mean (SEM).

RESULTS

DOPE Activates DGKα and DGKζ. Replacing DOPC with DOPE results in increased activity of both DGKα and DGKζ in both the presence and absence of Ca^{2+} (Figure 1). DGKα is known to be a calcium-dependent enzyme (*11*), in agreement with our findings at 0% PE (Figure 1A,B). However, most of the calcium sensitivity of DGKα is lost when assayed with liposomes containing 40% DOPE (Figure 1A,B). The activation of DGKα in the presence of DOPE without Ca^{2+} is proportional to the percentage of DOPE present in the liposomes (not shown). In contrast, the ζ-isoform shows a slight inhibition with the addition of 1 mM Ca^{2+} (Figure 1C,D) as we have previously observed using a mixed micelle assay (*9*).

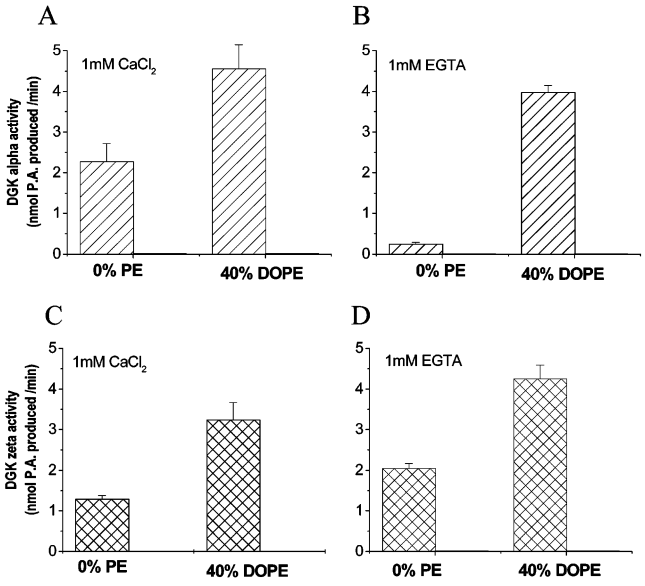

FIGURE 1: Comparison of DOPC and DOPE on the activity of DGKα and DGKζ in the presence and absence of Ca^{2+}. DGKα (A, B) and DGKζ (C, D) activities against liposomes containing 5% DOG, 40% DOPS, and 55% DOPC (0% PE) or 5% DOG, 40% DOPS, 15% DOPC, and 40% of DOPE (40% DOPE) in the presence of 1 mM $CaCl_2$ (A, C) or 1 mM EGTA (B, D). Assays using enzyme extracted from mock-transfected cells are plotted as bars to the right but are barely discernible at this scale. The assays were done in triplicate and confirmed in at least two independent experiments. The standard error of the mean (SEM) is plotted in all cases.

To assess if DGK activation by DOPE is due to a higher catalytic rate of the membrane-bound enzyme or to an increased partitioning of the enzyme to membranes, we performed a binding assay using sucrose-loaded vesicles (SLV). There is a greater binding of both DGK isoforms, α and ζ, to liposomes containing 40% DOPE compared with DOPC (0% PE) (Figure 2). The promotion of binding by DOPE is greatest for DGKα in the absence of calcium (Figure 2B). This behavior likely contributes to the greater activation by DOPE of DGKα in the absence of Ca^{2+} (Figure 1B). Thus either calcium or the presence of DOPE in the membrane will facilitate the binding of DGKα to membranes. On the other hand, DGKζ binding to membranes is inhibited by the presence of calcium, and this inhibition is reversed by the presence of DOPE in the membrane (Figure 2C,D).

DGK activity assays were also performed at high liposome concentration (12.7 mM) in order to approach complete translocation of the enzyme to the membrane. In this condition the binding of the enzyme to the membrane was between 83% and 98% (Table 1). The results of the activity measurements at high lipid concentration coincide with those at lower lipid concentration (4 mM) (Figure 1), showing greater activity in the presence of DOPE for both DGK isoforms (Table 1). The increase in enzymatic activity is greater than the increase in membrane binding (Table 1), indicating that the activation by DOPE is mediated both by an increase in membrane binding affinity and by a greater catalytic rate of the membrane-bound enzyme.

Cholesterol Activates DGKα and DGKζ. Cholesterol is a lipid structurally unrelated to PE, which also produces

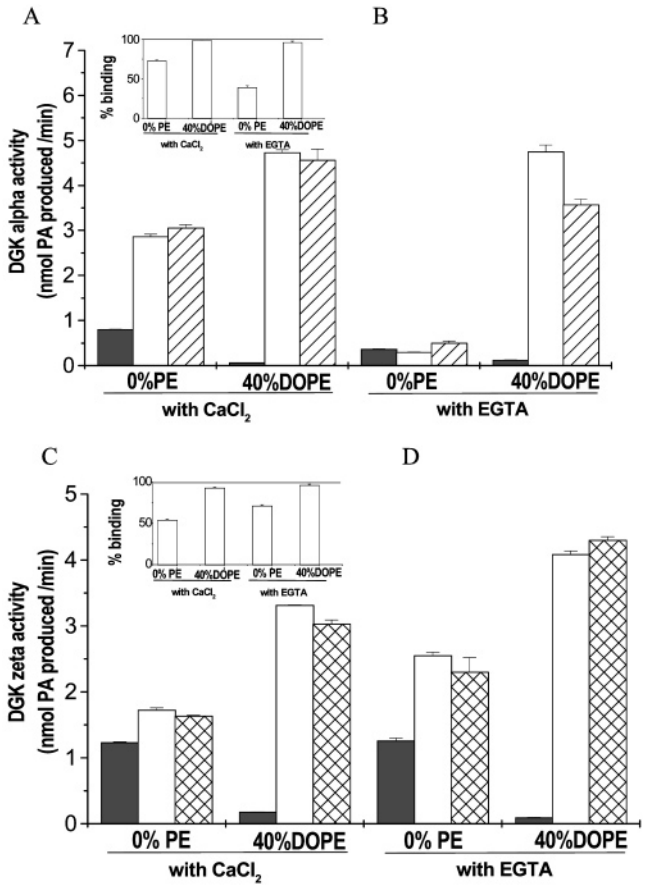

FIGURE 2: DGKα and DGKζ binding to sucrose-loaded vesicles (SLV). DGKα (A, B) and DGKζ (C, D) activities in unbound (gray bars), membrane-bound (white bars), and total prior to fractionation by centrifugation (striped bars) in the presence of 1 mM $CaCl_2$ (A, C) or 1 mM EGTA (B, D). The SLV lipid composition was 5% DOG, 40% DOPS, and 55% DOPC (0% PE) or 5% DOG, 40% DOPS, 15% DOPC, and 40% DOPE (40% PE). The insert shows the binding percentage in all cases. Binding was determined as the percentage of the total DGK activity in the bound fraction and was confirmed by Western blot using specific anti-DGK antibody. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

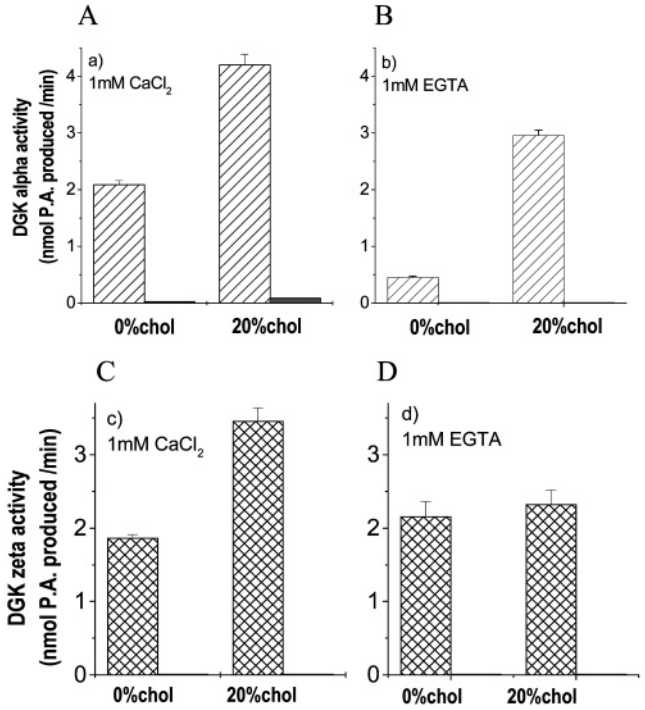

FIGURE 3: DGKα and DGKζ activity against liposomes containing cholesterol. DGKα (A, B) and DGKζ (C, D) activity against liposomes containing 5% DOG, 40% DOPS, and 55% DOPC (0% chol) or 5% DOG, 40% DOPS, 35% DOPC, and 20% cholesterol (20% chol) in the presence of 1 mM $CaCl_2$ (A, C) or 1 mM EGTA (B, D). Assays using enzyme extracted from mock-transfected cells are plotted as gray bars to the right but are not easily seen at this expansion. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

negative curvature stress (*16*). Like PE, cholesterol increases the activity of DGKα, particularly in the absence of calcium (Figure 3B). In contrast, cholesterol has little effect on DGKζ in the absence of calcium (Figure 3D), although it does activate this isoform in the presence of 1 mM Ca^{2+} (Figure 3C). Thus, although DGKζ normally does not require calcium for activation, its activity is increased by calcium with liposomes containing cholesterol. DGKζ does not contain a calcium-binding domain; however, it does contain a MARCKS homology domain that could bind to PS. Cholesterol may have different effects on DGKζ in the presence and absence of Ca^{2+} because of differences in electrostatic interactions caused by altering the dielectric constant at the membrane interface.

SLV binding results show significant increases (*t*-test, $p < 0.05$ in all cases) in the binding of both DGK isoforms to liposomes containing 20% cholesterol (Figure 4). Cholesterol induces the greatest increase (from 39 ± 5% to 70 ± 5%) in DGKα binding to the membrane in the absence of calcium. This behavior likely contributes to the observed increase in enzyme activity with the addition of cholesterol. It is notable that the ζ-isoform also shows a significant improvement in membrane binding with cholesterol, both in the presence of $CaCl_2$ ($p < 0.001$) and with EGTA ($p < 0.05$) (Figure 4B).

Table 1: DGKα and DGKζ Activity at High Lipid Concentration (12.7 mM) Using Liposomes Containing 5% DOG, 40% DOPS, and 55% DOPC (0% PE) or 5% DOG, 40% DOPS, 55% or 15% DOPC, and 40% DOPE (40% DOPE)[a]

	DGKα		DGKζ	
	0% PE	40% DOPE	0% PE	40% DOPE
fraction of enzyme bound to membranes[b]	0.83 ± 0.05	0.94 ± 0.03	0.90 ± 0.07	0.98 ± 0.01
% binding with DOPE[c]		113 ± 10		109 ± 10
activity (nmol of PA/min)	0.87 ± 0.10	4.3 ± 0.2	2.0 ± 0.3	4.1 ± 0.3
% enzymatic activity with DOPE[c]		496 ± 24		205 ± 13

[a] These experiments were performed at maximal activation conditions: 1 mM $CaCl_2$ (for DGKα) or 1 mM EGTA (for DGKζ). The assays were done in triplicate and confirmed in at least two independent experiments. The deviation is calculated as the standard error of the mean (SEM). [b] Determined by assay of DGK activity after separation of the membrane-bound fraction by centrifugation. The amount of enzyme bound is compared to the sum of the DGK activity of the bound and unbound fractions. Similar results are obtained by Western blotting. [c] Compared with the absence of DOPE.

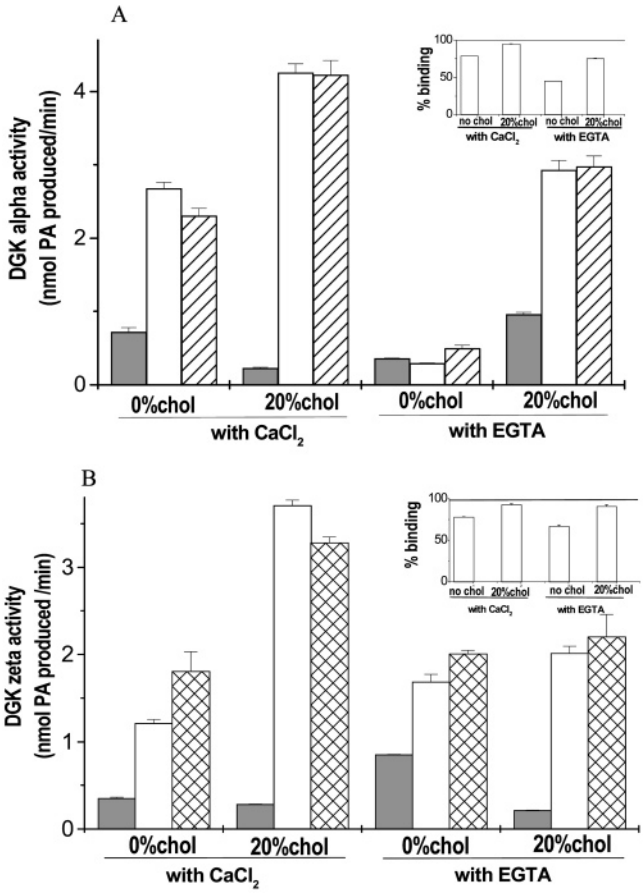

FIGURE 4: DGKα and DGKζ binding to sucrose-loaded vesicles containing cholesterol. DGKα (A) and DGKζ (B) activity of unbound (gray bars), bound (white bars), and total enzyme fraction (striped bars) using SLV containing 5% DOG, 40% DOPS, and 55% DOPC (0% chol) or 5% DOG, 40% DOPS, 35% DOPC, and 20% cholesterol (20% chol) in the presence of 1 mM $CaCl_2$ (left panel) or 1 mM EGTA (right panel). The insert shows the binding percentage in all cases. Binding percentages were determined as described in Figure 2. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

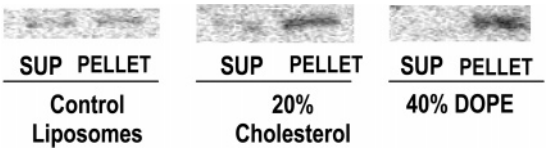

FIGURE 5: DGKα binding to liposomes containing DOPE or cholesterol by Western blot. The figure shows DGKα bands revealed by specific antibody of the unbound enzyme (SUP) and bound enzyme (PELLET) fraction of the SLV binding assay against control liposomes (55% DOPC, 40% DOPS, 5% DOG) or liposomes containing 20% cholesterol or 40% DOPE replacing DOPC. The binding experiment was developed in the presence of 1 mM EGTA. Binding rates calculated from scanning of bands using Scion Image software and from DGK activity assay (Figures 2 and 4) agree to within 10%.

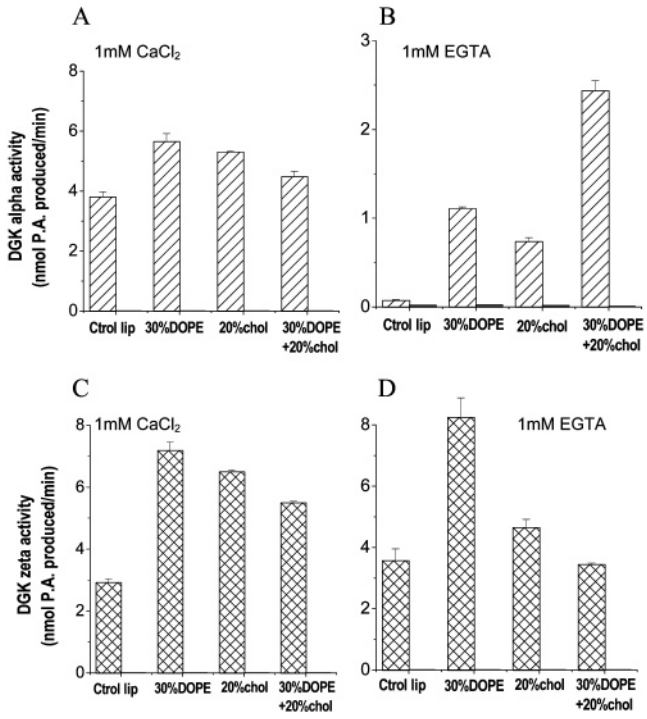

FIGURE 6: DGKα and DGKζ activities with liposomes containing DOPE and cholesterol. The activity of DGKα (A, B) and DGKζ (C, D) in the presence of 1 mM $CaCl_2$ (A, C) or in the presence of 1 mM EGTA (B, D) using liposomes containing 5% DOG and 30% DOPS as well as 65% DOPC (Ctrol lip), 30% DOPE and 35% DOPC (30% DOPE), 20% cholesterol and 45% DOPC (20% chol), or 30% DOPE, 20% cholesterol, and 15% DOPC (30% DOPE + 20% chol). Controls are plotted as gray bars (not observed on this scale). The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

Nevertheless, DGKζ activity in the presence of EGTA does not show a significant increase (Figure 3D), although the increased activity in the presence of Ca^{2+} is significant (Figure 3C). Thus the membrane binding and catalytic efficiency of DGKζ are differentially regulated by cholesterol.

There is some inaccuracy in the binding assays done by activity measurements as shown by the fact that in some cases the sum of the activities of the membrane-bound and soluble fractions is greater than the total activity measured without separation. We therefore confirmed the partitioning of the enzyme between the membrane and aqueous phase by analyzing the fractions from the SLV assay after centrifugation by directly measuring the amount of DGK in each fraction using Western blots. The increased partitioning of DGKα into the membrane fraction in the presence of either cholesterol or DOPE was confirmed (Figure 5).

DGK Activity with Liposomes Containing both DOPE and Cholesterol. The presence of both DOPE and cholesterol in the membrane produces a stimulation of DGKα activity in the absence of Ca^{2+} that is approximately equal to the sum of the activation caused by each of the components (Figure 6B). In the presence of calcium, DOPE plus cholesterol induces only a small activation, less than each of these lipid components individually (Figure 6A). Similarly, DGKζ activity in the presence of calcium is increased slightly less by the presence of both 30% DOPE and 20% cholesterol in the membrane than is found for either of these lipid components individually (Figure 6C). However, in the absence of calcium, the mixture of DOPE plus cholesterol inhibits DGKζ compared with the activity in the presence of either of these lipid components individually (Figure 6D). This is in contrast with DGKα that shows greater activity in the presence of DOPE plus cholesterol.

Dependence on Calcium Concentration. Our initial experiments, described above, have been done using 1 mM Ca^{2+}. To verify the physiological relevance of the effects of

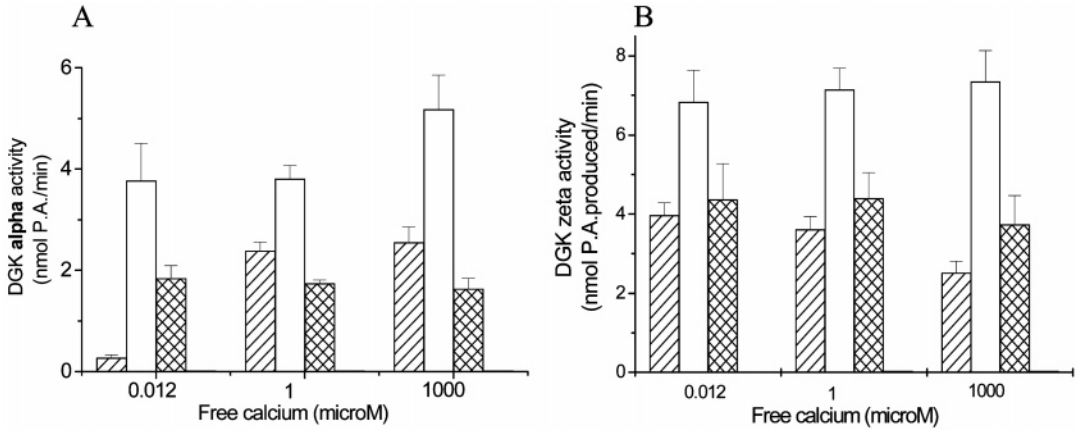

FIGURE 7: Effect of Ca^{2+} concentration on the activation of DGKα and DGKζ. The figure shows DGKα (A) and DGKζ (B) activity against liposomes containing 5% DOG and 40% DOPS and, in addition, 55% DOPC (diagonal stripped bars), 15% DOPC and 40% DOPE (white bars), or 35% DOPC and 20% cholesterol (diamond stripped bars) at different free calcium concentrations. This experiment was done at pH 7.2. Controls are plotted as gray bars but are not easily seen at this expansion. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

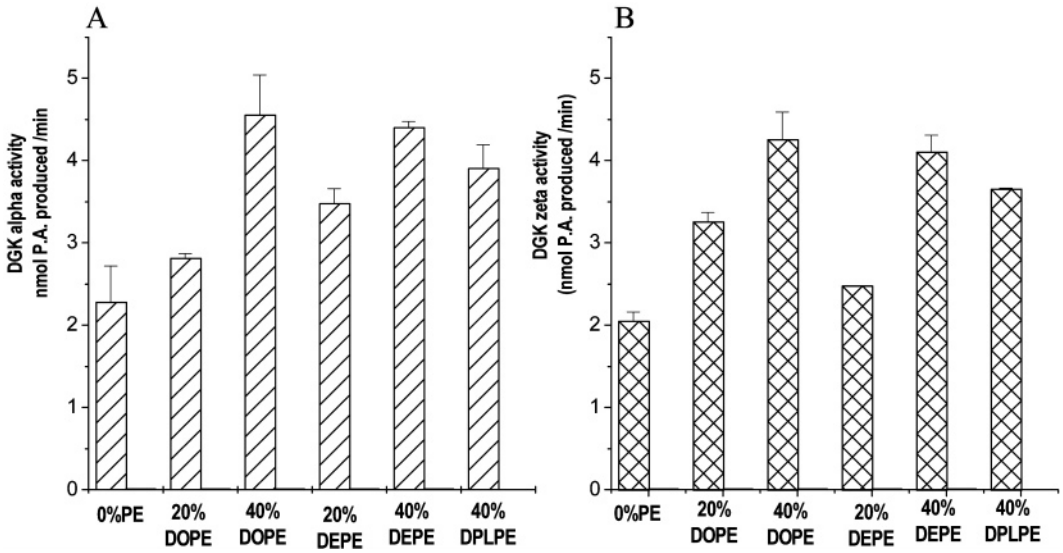

FIGURE 8: DGKα and DGKζ activity against liposomes containing different isomers of di-(18:1)PE. The figure shows DGKα activity in the presence of 1 mM $CaCl_2$ (A) and DGKζ activity in the presence of 1 mM EGTA (B). Activities were measured using liposomes containing 5% DOG, 40% DOPS, and 20% or 40% DOPE, DEPE, or DPlPE, as indicated, with the remaining lipid being DOPC. Controls are plotted as gray bars (below the resolution of the printed figure). The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

calcium that we have described, we have also measured the effects of DOPE and cholesterol at micromolar free calcium concentrations. These experiments were carried out at pH 7.2 instead of the usual pH 8 to obtain an accurate and constant concentration of free calcium. Without addition of either DOPE or cholesterol, DGKα exhibits close to maximal activation at 1 μM Ca^{2+} (Figure 7A, diagonal stripped bars). In contrast, DGKζ shows little dependence on Ca^{2+} at micromolar concentration in any of the conditions, although there is a small inhibition by Ca^{2+} in the millimolar range with the DOG/DOPC/DOPS mixture (Figure 7B). DGKα is dramatically activated by DOPE (13-fold) and cholesterol (6-fold) at the lowest free calcium concentration obtainable (12 nM), i.e., without the addition of Ca^{2+} (Figure 7A). Addition of higher concentrations of calcium has much less effect on the activity of DGKα in the presence of DOPE or cholesterol (Figure 7A). DOPE induces activation of DGKζ, but cholesterol has no effect (Figure 7B).

Mechanism of Lipid Modulation of the Activity of DGKα and DGKζ. Both lipids that promote the activity of DGKα and DGKζ, DOPE (*17*) and cholesterol (*16*), have intrinsic negative curvature. We further tested the relationship between monolayer curvature stress and activation of DGK using isomers of DOPE that should have similar properties, except for differences in their intrinsic curvature. The lipids were substituted for DOPE for either dielaidoylphosphatidylethanolamine (DEPE) or dipetroselinoylphosphatidylethanolamine (DPlPE). Both DEPE and DPlPE cause an increase in activity comparable to that observed with DOPE (Figure 8), despite the fact that these lipids have less negative curvature tendency. Membrane binding of both α- and ζ-isoforms of DGK, using SLV, is greater to liposomes containing either 40% DOPE or DEPE rather than DOPC at optimal calcium concentration conditions. The improvement of membrane binding induced by DOPE and DEPE is not statistically different according to a *t*-test (data not shown). Thus the

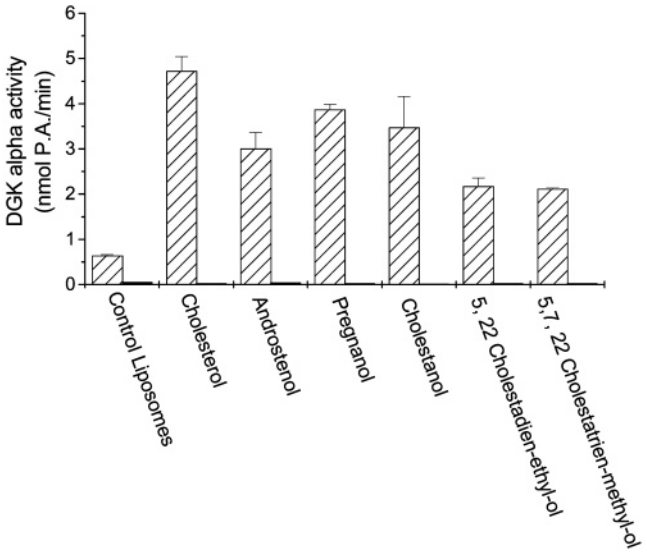

FIGURE 9: DGKα activity against cholesterol isomers. The figure shows DGKα activity against liposomes containing 5% DOG, 40% DOPS, and 55% DOPC (Control Liposomes) or with 20% sterol replacing a portion of the DOPC. The sterols used were cholesterol, androstenol, pregnan-3β-ol, cholestanol, 24β-ethyl-5,22-cholestadien-3β-ol, or 24β-methyl-5,7,22-cholestatrien-3β-ol, as described below each column. This experiment was done in the presence of 1 mM EGTA. Controls are plotted as gray bars but are not easily seen at this expansion. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

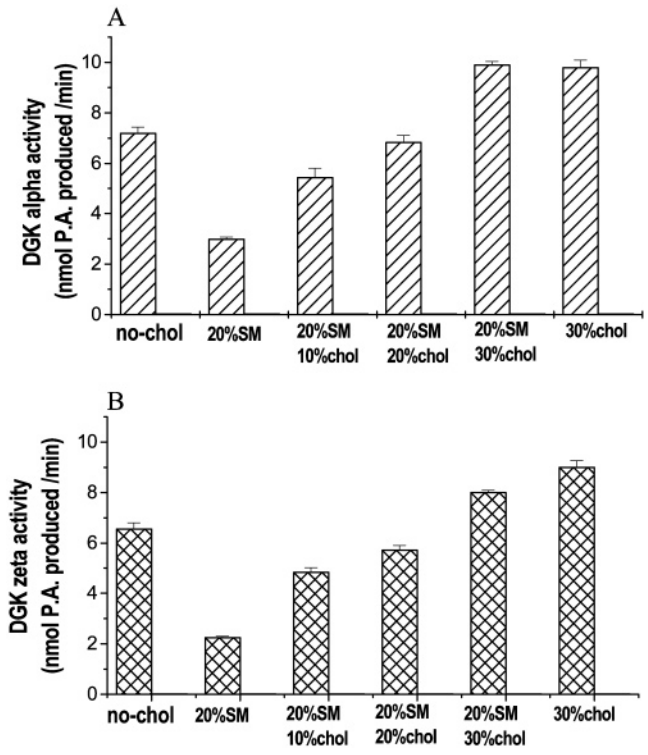

FIGURE 10: DGKα and DGKζ activities using liposomes containing sphingomyelin and cholesterol. DGKα (A) and DGKζ (B) activities using liposomes containing 5% DOG and 40% DOPS and, in addition, either 20% sphingomyelin (SM) and 35% DOPC (20% SM), 20% SM, 10% cholesterol, and 25% DOPC (20% SM, 10% chol), 20% SM, 20% cholesterol, and 15% DOPC (20% SM, 20% chol), 20% SM, 30% cholesterol, and 5% DOPC (20% SM, 30% chol), or 30% cholesterol and 25% DOPC (30% chol). 1 mM $CaCl_2$ was present in all cases. Controls are plotted as gray bars (below the resolution of the graph). The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

activation of DGKα and DGKζ by DOPE cannot be explained solely on the basis of membrane curvature.

We also studied the effect of other sterols on DGKα activity. The effect of the presence of 20% of five different sterols in the membrane results in a 3−6-fold activation in DGKα activity in absence of calcium (Figure 9). This effect is only slightly less than the 7-fold activation induced by 20% cholesterol.

Sphingomyelin alone inhibits both of the isoforms of DGK used in this study. It was known that sphingomyelin is less effective than several other lipids in supporting the activity of DGKα in the form of sonicated dispersions (*14*). This inhibition is reversed by the presence of cholesterol in the membrane (Figure 10).

Role of the E-F Hand in the Activation of DGKα by PE plus Cholesterol. DGKα is an 82 kDa DGK isoform that contains a regulatory region that includes tandem C1 domains and tandem Ca^{2+} binding E-F hand motifs as well as an N-terminal recoverin homology (RVH) domain that is related to the N-terminus of members of the recoverin family of neuronal calcium sensors. Two N-terminal deletion mutants of DGKα that lack one or more of the regulatory domains have been studied (*12*, *13*). DGKαΔ87 is a mutant that lacks the RVH regulatory domain but conserves the two E-F hand motifs and is capable of binding Ca^{2+}, while DGKαΔ196 lacks both the RVH and tandem E-F hands. This mutated form does not bind Ca^{2+} but exhibits significant calcium-independent activity (*13*). Our results using a liposome-based assay, without addition of DOPE or cholesterol, confirm the data from Jiang et al. (*13*) using a mixed micelle assay. The Δ196 mutant is constitutively active and is not further activated by calcium (Figure 11c and Table 2). However, we find that the activity of the wild-type enzyme is somewhat higher, in the presence of calcium, than that of DGKαΔ196. The Δ87 mutant shows a low activity in absence of calcium using liposomes without added DOPE or cholesterol (Figure 11b). Addition of Ca^{2+} to the Δ87 mutant results in a 4.4-fold activation (Figure 11b and Table 2). This activation was not observed using an octyl glucoside mixed micelle assay (*13*). However, even with the liposomal system used in the present work the activity of the Δ87 mutant is still lower than that of the wild-type enzyme even after the addition of calcium. The 4.4-fold activation by calcium of DGKαΔ87 is much less than the 20-fold found for the wild-type enzyme (Figure 11a and Table 2). Addition of 30% DOPE plus 20% cholesterol to the liposomes results in a 3.6-fold activation of the Δ87 mutant in the absence of calcium but no significant activation in the presence of calcium (Figure 11b, Table 2). In contrast, addition of 30% DOPE plus 20% cholesterol causes only a comparatively small activation of DGKαΔ196 in the presence of calcium (Figure 11b).

SLV-based binding assays were used to determine the partitioning of the mutant enzymes between the aqueous and membrane phases. Similar to the wild type, both mutant enzymes bind more to membranes containing 30% DOPE plus 20% cholesterol in the presence of calcium (Figure 12). The binding in the presence of calcium caused by the addition of 30% DOPE plus 20% cholesterol increases from 76 ± 3% to 97.75 ± 0.05% for the wild-type enzyme, from 77 ±

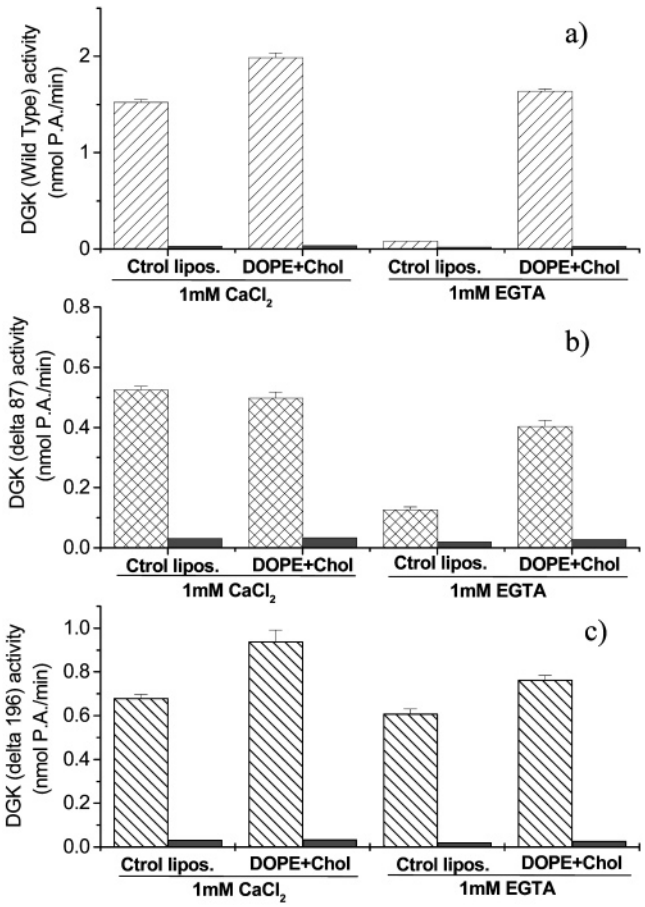

FIGURE 11: Activity of truncated DGKα mutants. Activity of wild-type DGKα (a), DGKαΔ87 (b), and DGKαΔ196 (c) using liposomes containing 5% DOG, 30% DOPS, and 65% DOPC (Ctrol lipos) or liposomes containing 5% DOG, 30% DOPS, 15% DOPC, 20% cholesterol, and 30% DOPE (DOPE + chol). The activities are normalized for the level of expression, as determined from Western blots. The experiments were done in the presence of 1 mM $CaCl_2$ (left panel) or 1 mM EGTA (right panel). Controls are plotted as gray bars but are not easily seen at this expansion. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

1% to 97.7 ± 0.5% for DGKαΔ87, and from 80 ± 1% to 99.4 ± 0.1% for DGKαΔ196. When the binding assay was carried out in the absence of calcium (1 mM EGTA), the extent of binding was generally lower, but the increase in binding with the addition of 30% DOPE plus 20% cholesterol was from 43.5 ± 0.5% to 99.5 ± 0.3% for the wild-type enzyme, from 74 ± 1% to 97.9 ± 0.2% for DGKαΔ87, and from 64 ± 1% to 99.2 ± 0.1% for DGKαΔ196.

DISCUSSION

Earlier studies on DGK activity using a mixed micellar assay system with detergents did not reveal any simple relationship between the physical properties of added phospholipids and their effect on the activity of DGKε or DGKζ (*9*). The present studies have used LUVs in the absence of detergent and reveal a marked activation of DGK by lipid components that promote negative curvature. Although some enzymes, such as protein kinase C (*18*), have shown effects of membrane curvature in micelle assays, which generally correlate with observed effects in bilayer systems, there are other enzymes in which this is not the case, such as CTP:

Table 2: Calcium Requirement for the Catalytic Activity of Mutant Forms of DGKα[a]

	EGTA		
	control liposomes (%)	DOPE + cholesterol (%)	*t*-test (*p*), $n = 3$
DGKα	4.9	104	0.001
DGKαΔ87	8	29	0.002
DGKαΔ196	44	53	0.05
	Calcium		
	control liposomes (%)	DOPE + cholesterol (%)	*t*-test (*p*), $n = 4$
DGKα	100	131	0.01
DGKαΔ87	35	33	0.34
DGKαΔ196	41	61	0.002

[a] Wild-type (DGKα), DGKαΔ87, and DGKαΔ196 activity with 5% DOG as substrate using liposomes, each of which contained 30% DOPS with the remaining lipid being either 65% DOPC (control liposomes) or 15% DOPC, 30% DOPE, and 20% cholesterol. These experiments were performed in the presence of either 1 mM $CaCl_2$ or 1 mM EGTA, as indicated. The activities are shown as a percentage of the activity compared with that of DGKα (wild type) using control liposomes in the presence of calcium (100%). The assays were done in triplicate and confirmed in at least three independent experiments. *t*-test results are shown for each pair, comparing activities with control liposomes and with liposomes containing DOPE + cholesterol.

phosphocholine cytidylyltransferase (*19*). There is no a priori reason to expect that the sensitivity of an enzyme to membrane curvature should be observed in micellar systems in which the curvature is dominated by the positive curvature tendency of the detergent. However, the presence of lipids that promote negative curvature also has other effects on membrane bilayers. It has been suggested, for example, that the enhancement of protein kinase C activity by lipid components that promote negative curvature is a consequence of altered interfacial properties (*20*).

In the present work we show that both DGKα and DGKζ have greater activity when part of the DOPC component of the membrane is replaced with DOPE. DOPE (*17*) promotes negative curvature. Cholesterol is another lipid component that promotes negative curvature (*16*), although its chemical structure and some of its physical properties are very different from those of DOPE. The promotion of activity by DOPE and by cholesterol is approximately additive in the case of DGKα in the absence of calcium, but addition of cholesterol lowers the activity of DGKζ observed with DOPE alone (Figure 6). This makes an interesting correlation with the cellular localization of these isoforms. Both DGKα and DGKζ can translocate to both the plasma membrane and the nucleus. We suggest that together PE and cholesterol, major lipid components of the cytoplasmic leaflet of the cell surface membrane, enhance the activity of these isoforms of DGK.

DGKα contains a calcium binding, E-F hand motif, and its activity had been found to be calcium dependent (*11*). We also find that with liposomes not containing DOPE or cholesterol this isoform is strongly activated by calcium (Figure 7, diagonal stripped bars). However, with the addition of either 40% DOPE or 20% cholesterol to the liposomes, DGKα can be converted from a calcium-dependent to a calcium-independent enzyme (Figure 7). The activity does not increase much further by raising the calcium concentration. The activation of DGKα at physiological pH and low free calcium concentration supports the conclusion that the

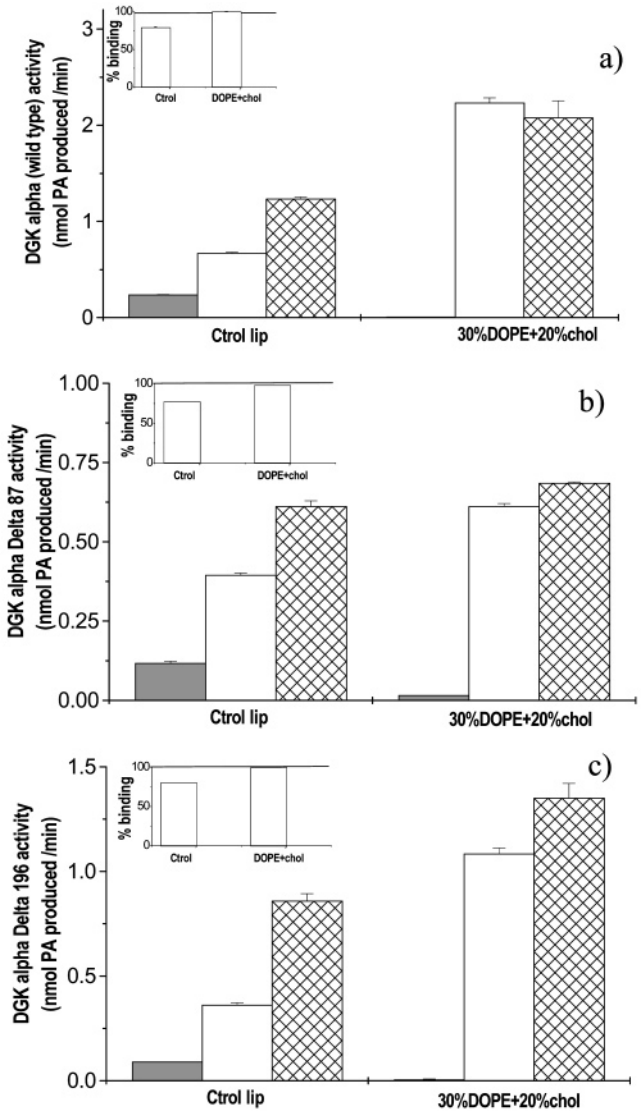

FIGURE 12: Binding to sucrose-loaded vesicles of truncated DGKα mutants. Wild-type DGKα (a), DGKαΔ87 (b), and DGKαΔ196 (c) activity of unbound (gray bars), bound (white bars), and total enzyme fraction (striped bars) against SLV containing 5% DOG, 30% DOPS, and 65% DOPC (Ctrol lip) or 5% DOG, 30% DOPS, 15% DOPC, 20% cholesterol, and 30% DOPE (30% DOPE + 20% chol) in the presence of 1 mM $CaCl_2$. The activities are normalized for the level of expression, as determined from Western blots. The insert shows the binding percentage in all cases. The binding percentage was determined as described in Figure 2. The assays were done in triplicate and confirmed in at least two independent experiments. The SEM is plotted in all cases.

fraction of DOPE and cholesterol in the region of the membrane that DGKα binds would play an important role in modulating the activity of this isoform.

We have investigated the mechanism responsible for the modulation of the activity of these two isoforms of DGK. The fact that both DOPE and cholesterol augment the activity of DGK illustrates that lipids that increase the propensity to form inverted phases are activators of DGK. It is not the actual formation of inverted phases that modulates the activity of the enzyme. Our liposomes are bilayers that are able to be extruded and to entrap sucrose, but the propensity to form inverted phases alters the physical properties of the membrane. To more critically evaluate the role of membrane intrinsic curvature, we substituted DOPE with two different isomers of this lipid. All three forms of PE have identical headgroups and lengths of acyl chains. However, DEPE has a trans rather than a cis double bond, and as a consequence the temperature of the transition to an inverted hexagonal phase is raised from about 8 °C for DOPE to 65 °C for DEPE. DEPE has less intrinsic negative curvature than DOPE (*21*). Despite the lower negative curvature properties of DEPE, this lipid has almost identical effects on DGK as does DOPE (Figure 8). Another di-(18:1)PE, DPlPE, has the position of unsaturation at carbon 6, rather than position 9 with DOPE or DEPE. DPlPE forms the hexagonal phase at 37 °C (*22*). This lipid also has an effect on DGK similar to that of DOPE (Figure 8). Quantitatively, however, DPlPE is slightly less potent than DOPE in activating both isoforms of DGK. This is the direction expected as a consequence of the differences in curvature stress, suggesting that this may be a contributing factor in the mechanism of activation, but the DEPE results clearly indicate that there is not a simple relationship between curvature stress and DGK activity. Hence, the presence of lipids that promote negative curvature strain increases the activity of DGK, but the mechanism of this effect is not due solely to changes in membrane curvature strain. DGK is not unique in this respect (*10*).

We also studied the effect of several sterols on DGKα activity (Figure 9). The results show that all five steroids analyzed are able to stimulate DGKα. These results demonstrate the lack of specificity of the cholesterol effect. In particular, androstenol has a significantly different chemical structure from cholesterol, and it is not capable of forming sterol-rich liquid-ordered domains (*23*). Nevertheless, androstenol activates DGKα about 60% as well as cholesterol.

Sphingomyelin inhibits the activity of DGK (Figure 10). Sphingomyelin has a high gel to liquid-crystalline phase transition temperature, near 37 °C. Unlike cholesterol and PE, sphingomyelin does not induce curvature stress. Addition of cholesterol, an activator of DGK, reverses the inhibition caused by sphingomyelin. It is known that sphingomyelin interacts strongly with cholesterol and can form segregated cholesterol-rich domains. Membranes having two or more coexisting phases that are physically separated into domains will have phase boundary defects in the locations where these domains meet. There are several cases in which the presence of such phase boundaries can lead to an increase in the activity of interfacial enzymes (*10*), as occurs, for example, with the enzyme sphingomyelinase (*24*). We find that the enzymatic activity is no greater in the presence of sphingomyelin and cholesterol than it is with the addition of only cholesterol. Thus, the presence of these putative domains in the liposomes containing both sphingomyelin and cholesterol does not result in an enhanced activity (Figure 10). In addition, although both cholesterol and sphingomyelin would be expected to decrease the fluidity of the membrane, one of these lipids is an activator and the other an inhibitor of DGK, suggesting, at least from the results of these two lipids, that fluidity is not a primary determinant of enzymatic activity.

In biological membranes there is evidence that sphingomyelin and cholesterol colocalize in caveolae and possibly in "raft" domains on the extracellular side of the plasma membrane. The physical state of these domains corresponds to a liquid-ordered phase. It is not known what the physical state and lipid composition of the cytoplasmic surface of

these domains are (i.e., the opposing monolayer), but it would not be surprising if there is a similar physical state on both sides of the bilayer, making our findings with mixtures of cholesterol and sphingomyelin of potential physiological relevance.

DGKα contains a calcium-binding E-F hand domain. However, it has been found that truncated mutant forms of DGK that lack the E-F hand domain are constitutively active in the absence of calcium (*12*, *13*). This has led to the suggestion that the amino-terminal segment blocks the active site of DGKα. One of the means of activating DGKα is suggested to be the binding of calcium to the E-F hand domain, triggering a conformational change that frees the active site. The presence of certain lipids in the membrane, such as DOPE and cholesterol, also leads to calcium-independent activity. These lipids also cause some activation of DGKαΔ87 in the absence of calcium but virtually no effect on DGKαΔ196 (Figure 11). Despite the lack of further activation of DGKαΔ196, the activating lipids cause increased translocation to the membrane for this truncated form as well as for DGKαΔ87 (Figure 12). Our results with "control" liposomes (Figure 11) are similar to those reported using a micellar assay for DGKαΔ196 (*13*) except that we observe a partial activation of DGKαΔ87 by calcium. It is not uncommon for liposomal assays to exhibit greater sensitivity to modulators of enzymatic activity, such as calcium, compared with micelle-based assays. This has been observed, for example, with protein kinase C (*25*). The observation that negative curvature lipids do not further promote the activity of wild-type DGKα in the presence of calcium, nor do these lipids further activate the constitutively active DGKαΔ196, suggests that DOPE and cholesterol activate the enzyme by inducing a conformational change that may remove the N-terminal region from the active site in a similar way that calcium does.

Some detergents also mimic the effects of PE and cholesterol in activating DGKα. It has been observed that the cationic amphiphile OTAC as well as the zwitterionic amphiphile C16SB activates DGKα (*13*, *26*). In the case of OTAC, there is no further activation of DGKα by the addition of calcium. With OTAC, the lack of calcium effect may be caused by electrostatic repulsion preventing access of calcium to the electrical double layer. Nevertheless, our findings of DOPE and cholesterol promoting calcium-independent activation of DGKα are not unique but provide an example of lipid components that can modulate the activity of DGKα in biological membranes.

Thus the activity and membrane binding of both DGKα and DGKζ are modulated by the lipid composition of the membrane. This will result in both modulation of the activity of the enzyme as a result of changes in the lipid composition of a biological membrane and changes in the subcellular localization of the enzyme. Membrane components that favor the formation of nonlamellar phases promote binding and activation of the enzyme. The mechanism of this effect, however, is not through a simple change in the intrinsic curvature of the membrane. Interestingly, activation of DGKα by DOPE and/or cholesterol results in the enzyme having maximal activity in the absence of calcium.

ACKNOWLEDGMENT

We are grateful to Mr. Matthew A. Churchward for assistance in developing the liposomal assay for DGK.

REFERENCES

1. Topham, M. K., and Prescott, S. M. (2002) Diacylglycerol kinases: regulation and signaling roles, *Thromb. Haemostasis 88*, 912−918.
2. Luo, B., Regier, D. S., Prescott, S. M., and Topham, M. K. (2004) Diacylglycerol kinases, *Cell Signalling 16*, 983−989.
3. 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.
4. 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.
5. 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.
6. Hogan, A., Shepherd, L., Chabot, J., Quenneville, S., Prescott, S. M., Topham, M. K., and Gee, S. H. (2001) Interaction of gamma 1-syntrophin with diacylglycerol kinase-zeta. Regulation of nuclear localization by PDZ interactions, *J Biol. Chem. 276*, 26526−26533.
7. Martelli, A. M., Manzoli, L., and Cocco, L. (2004) Nuclear inositides: facts and perspectives, *Pharmacol. Ther. 101*, 47−64.
8. Luo, B., Prescott, S. M., and Topham, M. K. (2004) Diacylglycerol kinase zeta regulates phosphatidylinositol 4-phosphate 5-kinase Iα by a novel mechanism, *Cell Signalling 16*, 891−897.
9. 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.
10. Epand, R. M. (2004) in *Structure of Biological Membranes* (Yeagle, P. L., Ed.) pp 493−503, CRC Press, Boca Raton, FL.
11. 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*, 59−64.
12. Abe, T., Lu, X., Jiang, Y., Boccone, C. E., Qian, S., Vattem, K. M., Wek, R. C., and Walsh, J. P. (2003) Site-directed mutagenesis of the active site of diacylglycerol kinase alpha: calcium and phosphatidylserine stimulate enzyme activity via distinct mechanisms, *Biochem. J. 375*, 673−680.
13. Jiang, Y., Qian, W., Hawes, J. W., and Walsh, J. P. (2000) A domain with homology to neuronal calcium sensors is required for calcium-dependent activation of diacylglycerol kinase alpha, *J. Biol. Chem. 275*, 34092−34099.
14. Kanoh, H., Kondoh, H., and Ono, T. (1983) Diacylglycerol kinase from pig brain. Purification and phospholipid dependencies, *J Biol. Chem. 258*, 1767−1774.
15. Ligeti, E., Dagher, M. C., Hernandez, S. E., Koleske, A. J., and Settleman, J. (2004) Phospholipids can switch the GTPase substrate preference of a GTPase-activating protein, *J. Biol. Chem. 279*, 5055−5058.
16. Chen, Z., and Rand, R. P. (1997) The influence of cholesterol on phospholipid membrane curvature and bending elasticity, *Biophys. J. 73*, 267−276.
17. Rand, R. P., Fuller, N. L., Gruner, S. M., and Parsegian, V. A. (1990) Membrane curvature, lipid segregation, and structural transitions for phospholipids under dual-solvent stress, *Biochemistry 29*, 76−87.
18. Epand, R. M. (1987) The relationship between the effects of drugs on bilayer stability and on protein kinase C activity, *Chem.-Biol. Interact. 63*, 239−247.
19. Cornell, R. B. (1991) Regulation of CTP:phosphocholine cytidylyltransferase by lipids. 1. Negative surface charge dependence for activation, *Biochemistry 30*, 5873−5880.
20. Giorgione, J. R., Kraayenhof, R., and Epand, R. M. (1998) Interfacial membrane properties modulate protein kinase C activa-

tion: role of the position of acyl chain unsaturation, *Biochemistry 37*, 10956−10960.
21. Keller, S. L., Gruner, S. M., and Gawrisch, K. (1996) Small concentrations of alamethicin induce a cubic phase in bulk phosphatidylethanolamine mixtures, *Biochim. Biophys. Acta 1278*, 241−246.
22. Epand, R. M., Fuller, N., and Rand, R. P. (1996) Role of the position of unsaturation on the phase behavior and intrinsic curvature of phosphatidylethanolamines, *Biophys. J. 71*, 1806−1810.
23. Waarts, B. L., Bittman, R., and Wilschut, J. (2002) Sphingolipid and cholesterol dependence of alphavirus membrane fusion. Lack of correlation with lipid raft formation in target liposomes, *J. Biol. Chem. 277*, 38141−38147.
24. Fanani, M. L., Hartel, S., Oliveira, R. G., and Maggio, B. (2002) Bidirectional control of sphingomyelinase activity and surface topography in lipid monolayers, *Biophys. J. 83*, 3416−3424.
25. Mosior, M., and Epand, R. M. (1994) Characterization of the calcium-binding site that regulates association of protein kinase C with phospholipid bilayers, *J. Biol. Chem. 269*, 13798−13805.
26. 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.

BI049145Z