Physiological Implications of the Contrasting Modulation of the Activities of the ϵ- and ζ-Isoforms of Diacylglycerol Kinase†

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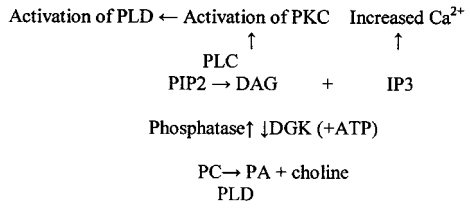
ABSTRACT: We have shown that the requirement of the ϵ-isoform of diacylglycerol kinase for diacylglycerols containing arachidonic acid is specific for this substrate and cannot be replaced by the presence of an arachidonoyl group in other places in the membrane; rather, it has to be present on the substrate itself. In addition, we demonstrate that the increased activity shown toward 1-stearoyl-2-arachidonoylglycerol by the ϵ-isoform of diacylglycerol kinase is not a consequence of altered membrane physical properties but is rather a specific interaction with the arachidonoyl group. We have also compared the modulation of the activity of the ϵ-isoform of diacylglycerol kinase with that of the ζ-isoform with regard to some of the intermediates involved in phosphatidylinositol cycling. One of the products of the hydrolysis of phosphatidylinositol diphosphate is diacylglycerol enriched in arachidonic acid. The activity of the ϵ-isoform is known to be specific for this form of diacylglycerol. We show that in contrast, the activity of the ζ-isoform is lower against 1-stearoyl-2-arachidonoylglycerol compared with dioleoylglycerol. We demonstrate that addition of phosphatidylserine, as well as other anionic phospholipids including L-α-phosphatidylinositol 4,5-bisphosphate, strongly inhibits the ϵ-isoform, but these anionic lipids increase the activity of the ζ-isoform. Addition of Ca^{2+}, which is released from internal stores as a consequence of phosphatidylinositol cycling, promotes the activity of the ϵ-isoform of this enzyme but has little effect on the ζ-isoform. The contrasting conditions required for maximal activity of these two isoforms of diacylglycerol kinase, as well as their different substrate specificity, suggest that they have different physiological roles in signal transduction.

One of the major pathways for signal transduction is through the cycling of phosphatidylinositols (PI).[1] The PI cycle is involved in gene expression, cell apoptosis, and many other intracellular regulatory functions. A first step in this process is the activation of PI-specific phospholipase C (PLC) isoforms that catalyze the conversion of L-α-phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 3′,4′,5′-trisphosphate (IP3). Both the DAG and the IP3 products serve as important secondary messengers. One of the functions of DAG is to activate the enzyme protein kinase C as well as other proteins. DAG can be produced either by phospholipase C-catalyzed hydrolysis of PIP2, as described above, or by the action of phospholipase D to produce phosphatidic acid (PA), followed by the action of a lipid phosphatase. This can result in an autocatalytic loop in which diacylglycerol produced through the phospholipase D (PLD) path increases DAG levels which in turn stimulates protein kinase C (PKC), which is known to activate phospholipase D (*1*) by phosphorylation. This is summarized in the simplified scheme below:



However, in cell systems, it is primarily the more highly unsaturated DAG formed from PIP2 that activates protein kinase C (*2, 3*). Control of the concentration of DAG in the membrane is an important component of the regulation of signal transduction. The enzyme diacylglycerol kinase (DGK) plays a major role in removing DAG by catalyzing its phosphorylation using ATP as the phosphate donor (*4*).

There are at least nine different isoforms of mammalian DGK, which have been separated into five classes on the basis of their different structures and properties (*5, 6*). All

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[1] Abbreviations: BHT, butylated hydroxytoluene; DAG, diacylglycerol; DAPC, 1,2-diarachidonoylphosphatidylcholine; DAPE, 1,2-diarachidonoylphosphatidylethanolamine; DGK, diacylglycerol kinase; DOG, 1,2-dioleoyl-*sn*-glycerol; DOPA, 1,2-dioleoylphosphatidic acid; DOPC, 1,2-dioleoylphosphatidylcholine; DOPE, 1,2-dioleoylphosphatidylethanolamine; DOPI, 1,2-dioleoylphosphatidylinositol; DOPS, 1,2-dioleoylphosphatidylserine; DTT, dithiothreitol; IP3, inositol 1,4,5-trisphosphate; NP-40, nonaethylene glycol octaphenyl ether; OG, octyl glucoside; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP2, L-α-phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol.

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of them contain a conserved catalytic domain and at least two cysteine-rich C1 domains. Most DGKs are expressed in the brain, but some, like DGKϵ, show more ubiquitous expression. These isozymes differ from each other predominantly by their substrate specificity, tissue distribution, and regulation. DGKζ is found in the nucleus, where it can reduce the amount of nuclear diacylglycerol resulting in the attenuation of cell growth (*7*). This isoform also regulates Ras activation (*8*).

In this work, we studied two of the DGK isoforms, ϵ (*9*) and ζ (*10*). DGKζ is a 117 kDa protein that has a MARCKS homology domain. This domain in the MARCKS protein can interact with PIP2 (*11*), suggesting DGKζ may also bind to PIP2, or perhaps to other negatively charged phospholipids. DGKϵ is also of interest because it is the only DGK that exhibits apparent specificity for DAGs with arachidonic acid (*9*) and other polyunsaturated acyl chains (*2*). This isoform regulates seizure susceptibility and long-term potentiation (*12*). It has been observed in cells that free arachidonic acid can promote the translocation of DGKα and DGKγ to a membrane (*13*), suggesting some specific interaction of other isoforms of DGK with an arachidonate moiety. Alternatively, the arachidonic acid may have initiated other signaling mechanisms that induced the translocation.

Changes in intracellular Ca^{2+} levels are also associated with phosphatidylinositol cycling because the product IP3 can bind to ligand-gated channels in the endoplasmic reticulum, leading to the intracellular release of calcium stores. It is known that the α-, β-, and γ-isoforms of Type I DGK have EF-hand domains that can bind to Ca^{2+}, resulting in the activation of the enzyme (*5*), and its translocation to membranes (*14*). The effects of Ca^{2+} on other DGK isoforms have not been extensively tested, but most of them seem to be independent of Ca^{2+} (*15*). Other potential modifiers of DGK activity are negatively charged phospholipids present in the membrane bilayer. One of these, phosphatidylserine, is important for the binding of certain proteins to membranes. Phosphatidylserine has also been shown to enhance the activity of some DGK preparations (*16*). Another anionic membrane lipid, PIP2, is the precursor of DAG formed from hydrolysis of PIP2 and predominantly exists in the cell as the 1-stearoyl-2-arachidonoyl species (*17*, *18*) that will yield *sn*-1-stearoyl-2-arachidonoylglycerol (SAG), the preferred substrate of DGKϵ.

Although DGK has been purified from natural sources (*15*), no single mammalian isoform has been purified to homogeneity. In the present work, we have studied the enzymatic properties of isoforms DGKζ and -ϵ by overexpressing the proteins in insect cells. This allowed for larger amounts of protein to be expressed than is possible with current mammalian expression systems. The DGK produced as a result of transfection is far in excess of the endogenous DGK activity. These preparations therefore have essentially only the DGK activity of the transfected isoform. However, the level of expression of the transfected DGK, although fairly reproducible, is not known. Therefore, the measured activities should be considered as relative activities, and the relationship of the observed activities of DGKϵ vs DGKζ is not meaningful. We have used a detergent-solubilized micellar system to study the activity of DGK. Previous studies with protein kinase C demonstrated that membrane additives that modulated the activity of protein kinase C exhibited similar effects when assayed with a Triton-solubilized assay system as when the assays are performed with liposomes (*19*). This work represents the first direct comparison between two specific isoforms of DGK. The results demonstrate that the conditions required for their maximal activity are very different, suggesting that they have different physiological roles, despite being isoforms of the same enzyme.

EXPERIMENTAL PROCEDURES

Materials. The lipids PIP2 and SAG were purchased from Biomol (Plymouth Meeting, PA). Other phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Histone HI was from GIBCO/BRL (Grand Island, NY); ATP was the disodium salt, SigmaUltra grade (Sigma, St. Louis, MO). [γ-^{32}P]ATP was from ICN. Other chemicals were of the purest grade available. Double distilled water was used for all solutions.

Enzyme Preparations. cDNAs encoding either DGKϵ or DGKζ were cloned into BacPAK6 (Clontech), and baculovirus stocks were generated using the BakPAK system (Clontech) according to the manufacturer's instructions. Sf21 cells were infected with the virus stocks, and then the cells were harvested 72 h later in 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP40, 1 mM DTT, 1 mM PMSF, 0.5 mM sodium orthovanadate, and 10 mg/mL each of leupeptin, pepstatin, aprotinin, and soybean trypsin inhibitor. They were allowed to lyse for 10 min and then centrifuged to remove debris. The supernatants or mock-infected supernatants were then used for the assays.

Assay of DGK Activity. Our assay was adapted from the procedure described by Walsh et al. (*20*). Briefly for each assay, 4 μL of a DAG stock solution (DOG or SAG, both 40 mM in 1:1 $CHCl_3/CH_3OH$, stored at -20 °C under argon) together with any other lipid component being used in the assay was dried under N_2 in a silanized glass test tube and then placed under vacuum for 2 h to remove the last traces of solvent. The lipid was hydrated with 50 μL of 4× assay buffer (60 mM Triton X-100, 200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 20 mM $MgCl_2$, 4 mM EGTA, and 30 μg/mL BHT; and with or without the addition of 10 mM $CaCl_2$), 2 μL of 100 mM DTT, 1 μL of 1 mg/mL histone, and 5 μL of cell lysate either from cells transfected with the DGK enzyme or from mock-transfected cells. The final volume was 180 μL. The reaction was initiated by adding 20 μL of 5.0 mM [γ-^{32}P]ATP (75 μCi/mL). The final assay mixture contained 15 mM Triton X-100, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.75 μg of BHT, 3.5 mM phospholipid (except where otherwise noted), 1 mM DTT, 5 μg/mL histone, 0.80 mM DAG, and enzyme. The reaction was carried out for 10 min at 25 °C and was terminated by the addition of 2 mL of $CHCl_3$/methanol (1:1 v/v) containing 0.25 mg/mL dihexadecyl phosphate. The organic phase was washed 3 times with 2 mL each of 1% $HClO_4$, 0.1% H_3PO_4 in H_2O/methanol (7:1 v/v). The volume of the final $CHCl_3$ phase was 0.80 mL. A 0.50 mL aliquot of the organic phase was dried under N_2, and the incorporation of ^{32}P into PA was determined by Cerenkov counting. Controls were run without the addition of enzyme or with the addition of mock-transfected cell lysates. In both cases, the counts remaining in the organic phase were not signifi-

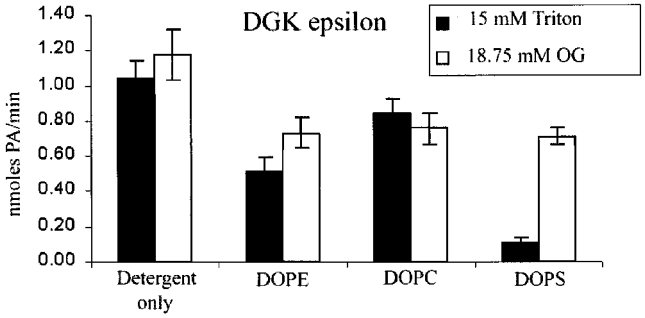

FIGURE 1: Comparison of effects of added phospholipids on the activity of DGKϵ using octyl glucoside vs Triton X-100 micelles. Micelle-based assays contained either 15 mM Triton X-100 (black bars) or 18.75 mM octyl glucoside (OG, white bars) in the buffer 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, and 0.75 μg of BHT, 1 mM DTT, 0.5 mM ATP, 5 μg/mL histone, and 5 μL of cell lysate. 0.8 mM 1,2-diolein was used as substrate in all cases. The first two bars correspond to the absence of added phospholipid, and subsequent bars are for samples to which 3.5 mM of the indicated lipid was added. Formation of the product, phosphatidic acid (PA), was determined after extraction by the incorporation of ^{32}P. The counts were corrected for the DGK activity of noninfected cells.

cantly above the background level. The production of PA was linear with time over 10 min. A variation of this assay, but utilizing octyl glucoside (OG) micelles, was carried out in an analogous manner to that described above for the Triton micelles, except for the following replacements: 15 mM Triton X-100 for 18.75 mM OG; 50 mM Tris-HCl, pH 8.0, for 50 mM MOPS, pH 7.2; 20 mM for 5 mM $MgCl_2$. These changes follow those previously used (*20*).

The assays were done in triplicate and the results presented with errors showing the standard deviation of the mean for one particular experiment. Each experiment was independently repeated at least twice. The day to day variations using the same enzyme preparation and the same lipids were not much greater than those for an individual experiment. However, comparison of activities measured with different batches of transfected cells or with cell pellets stored for more than 6 months at −80 °C showed greater variation, but the effects of added substances were similar.

RESULTS

Changes in the Rate of Phosphorylation by DGK with Added Phospholipids. It has previously been shown that the specificity of certain forms of DGK for the arachidonoyl group is exhibited in several detergent micelle systems (*20*). We have compared the action of several phospholipids on the rate of phosphorylation of 1,2-dioleoyl-*sn*-glycerol (DOG) by DGKϵ in both the octyl glucoside (OG) micellar assay system as well as the Triton X-100 assay system (Figure 1). Both zwitterionic (DOPE and DOPC) lipid as well as the anionic lipid 1,2-dioleoylphosphatidylserine (DOPS) inhibit this reaction. The inhibition by DOPS is the greatest of the three phospholipids, but is much more pronounced in the Triton micelle system than in the OG assay. Attempts to carry out these experiments in membrane bilayer systems were not successful, and the conditions required for this type of assay have not yet been established.

Comparison of Substrate Specificity in OG vs Triton Micelles. The relative rates of phosphorylation of DOG and SAG are similar in the OG and Triton micelle assays for

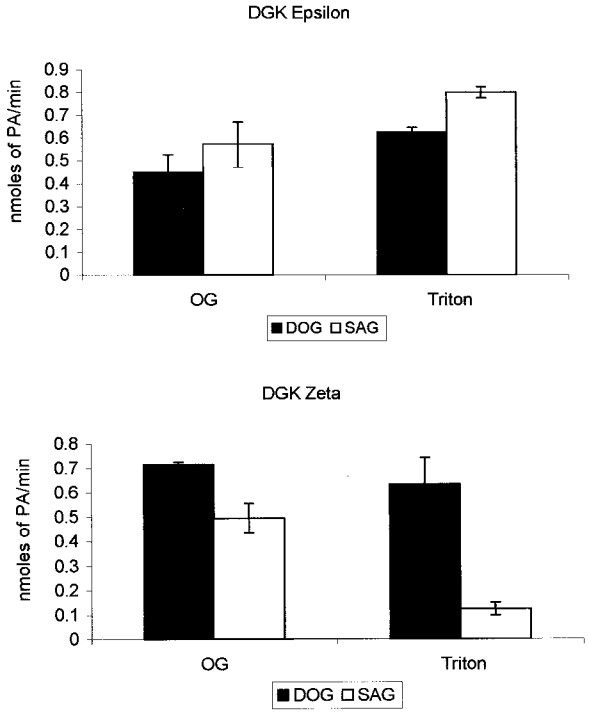

FIGURE 2: Comparison of the OG and Triton assay systems. Conditions similar to Figure 1 without the addition of phospholipid. Phosphorylation was measured using one of the two substates: 1,2-diolein (DOG, black bars) or 1-stearoyl-2-arachidonylglycerol (SAG, white bars).

both DGKϵ and DGKζ (Figure 2). These assays were done at a later time than those presented below using a different batch of SAG and of DGKϵ. Other results shown below indicate a greater specificity of DGKϵ for SAG vs DOG. The major conclusion from this assay is that OG and Triton assay systems produce similar, but not quantitatively identical, results.

Acyl Chain Dependence of Phospholipid Modulation of DGK Activity. DGKϵ more rapidly phosphorylates DAGs containing an arachidonoyl group. We investigated if this selectivity is a consequence of effects of the arachidonoyl group on nonspecific physical properties and, in addition, if the requirement for an arachidonoyl group in the substrate could be substituted by having it on other membrane lipids. We have compared the modulation of the activity of both the ϵ- and the ζ-isoforms of DGK (Figure 3), using either DOG or SAG as substrate with several added zwitterionic lipids having different degrees of unsaturation in one or both of the acyl chains as well as different headgroups. One of the physical properties that differ among these lipids is their intrinsic membrane curvature. There are many examples demonstrating that membrane curvature modulates certain functions of biological membranes (*21*). Either sterically smaller headgroups or increased acyl chain unsaturation are among the factors that will increase the negative curvature tendency of the lipids. Changing from a lipid such as POPC to one that has both increased unsaturation as well as a small headgroup, such as DAPE, has a relatively modest effect on the activity of DGK, although it is slightly inhibitory for DGKϵ with a weak potency. We conclude from these experiments that the specificity of DGKϵ for the arachidonoyl group is not likely to be a consequence of altered physical properties. This is an important distinction because DAG can

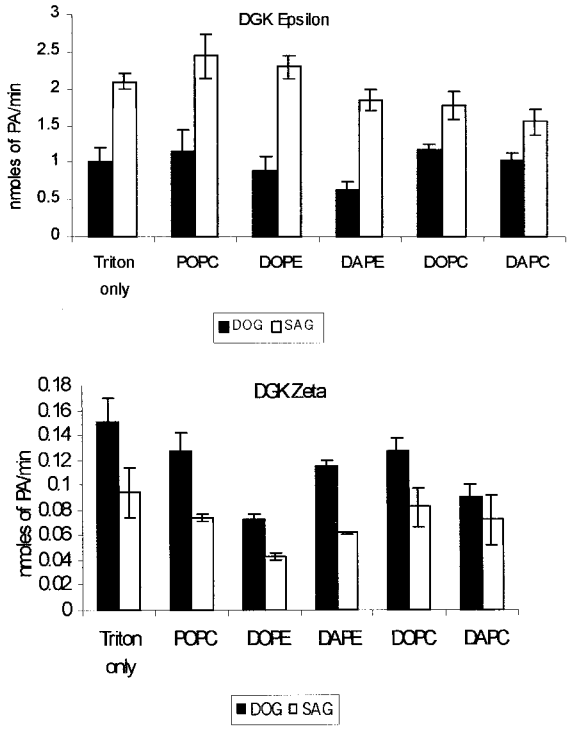

FIGURE 3: Dependence of DGKϵ or DGKζ activity on the acyl chain composition of added phospholipids. The assays were carried out using Triton X-100 comicelles as described in the legend of Figure 1. No phospholipid was added to the "Triton only" samples. For the other cases, 3.5 mM of the indicated phospholipid was added. Phosphorylation was measured using one of the two substates: 1,2-diolein (DOG, black bars) or 1-stearoyl-2-arachidonylglycerol (SAG, white bars).

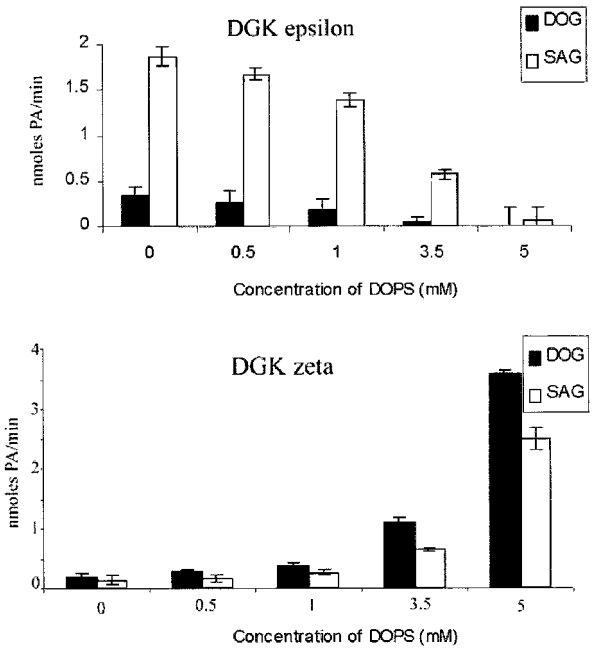

FIGURE 4: Effect of DOPS on DGKϵ and DGKζ activity. Conditions as for Figure 3 except that the added phospholipid is DOPS at a series of increasing concentrations.

differentially activate signaling pathways depending on its acyl chain composition (*2, 3*). Thus, through its enzymatic activity, DGKϵ could preferentially terminate distinct signaling pathways, which may be a general mechanism for DGKs to regulate specific DAG signals.

Dependence on DOPS. Walsh et al. (*20*) found that negatively charged phospholipids (PS, PA, and PIP2) variably affected several different DGKs, including an arachidonoyl-specific one that they had partially purified. However, because the enzyme preparations that they used were only partially purified, they could not specifically identify the DGK isozymes that they studied. We have compared how negatively charged lipids affected the activities of DGKϵ and DGKζ. We found that DGKϵ activity decreases with increasing DOPS concentration (Figure 4). This suggests that the arachidonoyl-specific DGK in those studies (*20*) may have been DGKϵ or that this may be a generalized property of both DGKϵ and other unidentified type III DGKs. In contrast, DGKζ activity increases with increasing DOPS concentration (Figure 4) with both DOG and SAG. This effect of DOPS is observed in both the Triton and the OG assay systems (Figure 5). This is the first example of a DGK showing activation in the presence of an anionic lipid. These data demonstrate the complexity of the DAG kinase family and suggest that these enzymes are differentially regulated by components of the membrane microenvironment.

Effect of Other Anionic Lipids. To determine the specificity of the effects of phosphatidylserine on the activity of DGK, we also measured the effect of two other anionic lipids, 1,2-dioleoylphosphatidylinositol (DOPI) and 1,2-dioleoylphosphatidic acid (DOPA). Addition of DOPI or DOPA decreases the activity of DGKϵ but increases the activity of DGKζ (Figure 6) using either DOG or SAG as substrate, indicating that the effect of anionic lipids is likely a nonspecific electrostatic effect. DGKζ has a MARCKS homology domain, which is very cationic and may interact strongly with negatively charged lipids such as phosphatidylserine. Thus, it is possible that a component of the activation of DGKζ may be caused by the interaction of its MARCKS domain with negatively charged lipids within the membrane.

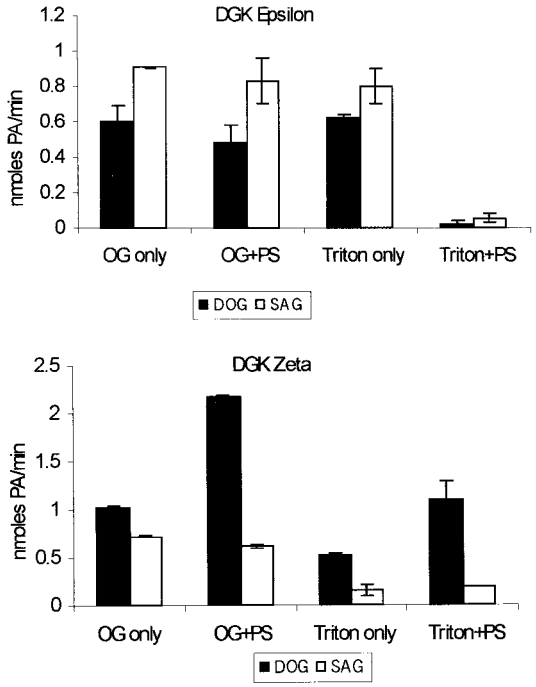

FIGURE 5: Comparison of the effect of DOPS on DGKϵ and DGKζ activity using either the OG- or the Triton-based assay system. Conditions as for Figure 2 except that DOPS was added where indicated at a concentration of 10 mM.

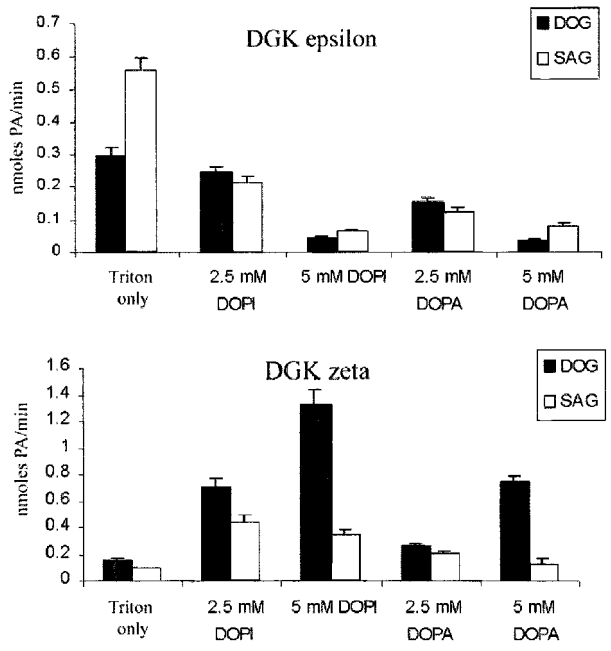

FIGURE 6: Effect of DOPI and DOPA on DGKϵ and DGKζ. Conditions as for Figure 4 except that the added phospholipid is either DOPI or DOPA at a concentration of 2.5 or 5 mM.

In addition, DGKϵ has a cluster of cationic Arg residues at positions 44, 46, 47, 51, 52, and 56 which is adjacent to a putative transmembrane helix comprising approximately residues 20−40 (*22*). This cationic cluster may also interact with anionic lipids.

Dependence on Calcium Concentration. Upon activation of several types of membrane receptors, PI-specific PLC isoforms initiate DAG signaling. The other product released by their activity, IP3, causes calcium release from intracellular stores. Because of the simultaneous generation of DAG and IP3, activation of DGK activity by calcium would be an elegant mechanism to precisely control DAG signaling cascades. Calcium is known to activate type I DGKs, but its effect on other isoforms has not been extensively characterized. We tested the effect of Ca^{2+} on DGKϵ and DGKζ activity both in the presence and in the absence of 5 mM DOPS (Figure 7). DGKϵ is activated significantly in the presence of 2.5 mM calcium chloride, but 5 mM DOPS inhibits this stimulation. Calcium has little effect on the activity of DGKζ, and 2.5 mM Ca^{2+} does not prevent the activation of DGKζ by 5 mM DOPS. The activation of DGKϵ but not DGKζ by calcium is not surprising since the diacylglycerol, formed from PIP2, is enriched in arachidonic acid and would be a preferred substrate for DGKϵ. Thus, activation of DGKϵ by calcium could provide a negative feedback loop to terminate the DAG signal. The concentration of Ca^{2+} used is well above physiological concentrations. We have found that there is also significant inhibition at 1 mM Ca^{2+} but not at 100 μM Ca^{2+}. It should be noted that the sensitivity of protein kinase C to Ca^{2+}, in the absence of diacylglycerol, is much lower in a Triton micelle assay than it is in a phospholipid vesicle assay (*23*). We suggest that the requirement for a high concentration of Ca^{2+} to activate DGKϵ is a consequence of the assay system and it is not observed with DGKζ.

Effect of PIP2. PIP2 is a precursor of DAG. Walsh et al. (*24*) demonstrated that PIP2 inhibited a partially purified

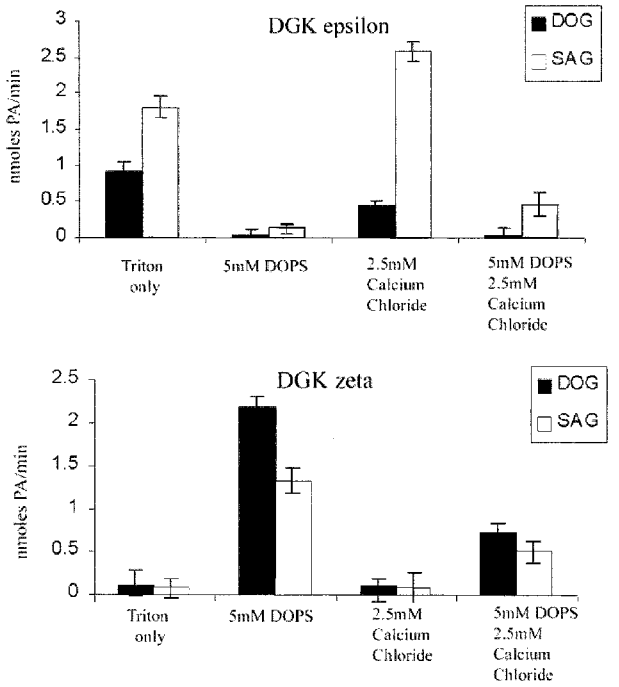

FIGURE 7: Effect of calcium ions on DGKϵ and DGKζ activity. The assays were carried out using Triton X-100 comicelles as described in the legend of Figure 1. No phospholipid was added to the "Triton only" sample. Other conditions include the addition of 5 mM DOPS (no added calcium), 2.5 mM $CaCl_2$ (no added phospholipid), or 5 mM DOPS with 2.5 mM $CaCl_2$. Phosphorylation was measured using one of the two substates: 1,2-diolein (black bars) or 1-stearoyl-2-arachidonylglycerol (white bars).

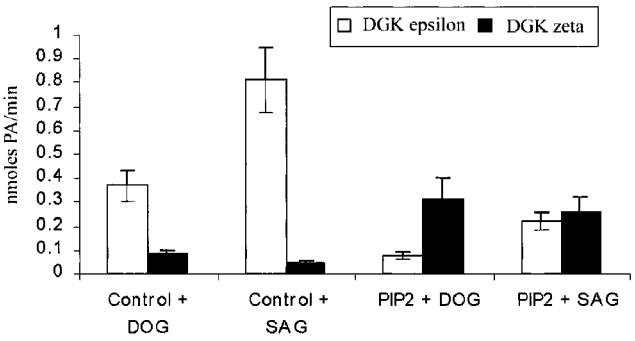

FIGURE 8: Effect of PIP2 on DGKϵ and DGKζ activity. Assays with Triton micelles and no added phospholipid are indicated as "Control". The two pairs of bars on the right have 0.24 mM PIP2 added. The diacylglycerol concentrations are 0.8 mM. Either DGKϵ (white bars) or DGKζ (black bars) was used.

DGK that preferred arachidonoyl-DAG as a substrate. However, they could not definitively identify the DGK isotype that they studied. We determined the effect of PIP2 on DGKϵ and DGKζ (Figure 8). We found that PIP2 (0.24 mM) inhibits DGKϵ, in agreement with the findings using the arachidonoyl-specific DGK from bovine testes (*24*). As PIP2 is the precursor of arachidonoyl-DAG, the preferred substrate of DGKϵ, its inhibition of DGKϵ may represent another mechanism to tightly control DAG kinase activity in the membrane microenvironment. Conversely, PIP2 increased the activity of DGKζ. This activation is consistent with our previous data (Figures 4 and 6). It is expected that the MARCKS domain of DGKζ would have particularly strong interactions with PIP2 (*25*) and other anionic phospholipids.

DISCUSSION

In this work we have used a micelle-based assay system. Our results indicate that the principle factors modulating the activity of DGK are not dependent on the nature of the assay system and would therefore also be observed in a bilayer-based assay. This is supported by the fact that we obtain similar effects of added lipids in the OG- and Triton-based assay systems, although there are some quantitative differences (Figures 1, 2, and 5). Furthermore, the effect of different zwitterionic lipids on the activity of the enzyme in Triton micelles is relatively modest, despite the large differences in the physical properties of these lipids (Figure 3). This indicates that the enzyme is generally not very sensitive to the nature of the surrounding lipids, if the lipid does not interact directly with the DGK. In the case of the DAG substrate, there will be specific interactions with the DGK that can affect the activity directly or indirectly through changes in the physical properties of the membrane environment of the enzyme.

DGKϵ has greater activity in phosphorylating arachidonoyl-DAG compared with other diacylglycerols. This difference is not a consequence of microenvironmental changes in the membrane caused by arachidonic acid, since addition of arachidonate-rich PC or PE to the micelles does not significantly change the activity of DGKϵ (Figure 3). This is consistent with observations with the DGK isolated from testes whose inhibition by PA is not sensitive to the presence of an arachidonoyl group in the *sn*-2 position (*24*). It should also be noted that the specificity of DGKϵ for arachidonoyl-DAG is relative and not absolute, since 1,2-dioleoyl-*sn*-glycerol is also phosphorylated generally at about 10−50% of the rate of SAG, depending on the conditions. A factor that may contribute to the higher rate of phosphorylation of SAG may be its tendency to cluster in membranes (*26*), although this factor should be of less importance in the assay systems we have employed.

Zwitterionic lipids had little effect on the activity of DGK (Figure 3), and the small effects observed were largely independent of whether the lipid was PC or PE or the degree of unsaturation of the acyl chains. Earlier studies with arachidonoyl-DGK isolated from bovine testis (*20*) showed inhibition with PE and slight stimulation with PC, particularly at mole fractions higher than the 6 mol % used here. The quantitative affects of the added phospholipid also depend on the nature of the acyl chain (Figure 3); however, the differences are small. The small differences observed do not correlate in a simple manner with any physical property of the lipid. This behavior is different from that of many other interfacial enzymes (*27*) including protein kinase C (*28*), phospholipase A2 (*29*), diglucosyl diacylglycerol synthase (*30*), or CTP phosphocholine cytidyltransferase (*31*), which are sensitive to the intrinsic curvature properties of the membrane. The insensitivity of DGKϵ to changes in the bulk physical properties demonstrates that this is not a mechanism to explain its specificity for arachidonoyl-DAG.

In contrast to zwitterionic lipids, DOPS has a marked effect on the activity of the two DGK isoforms we have studied. Interestingly, the effects on the two isoforms are opposite, with DGKζ being activated by PS and DGKϵ being inhibited (Figure 4). This is observed in both the OG and Triton assay systems (Figure 5). Arachidonoyl-DGK isolated from bovine testis was previously shown to exhibit inhibition by PS (*20*). When combined with the earlier observation that DGKϵ has specificity for arachidonic acid (*9*), this indicates that if one can extrapolate from this in vitro system to that of a cell, these two DGK isoforms, ϵ and ζ, may have different functions in vivo by virtue of this differential regulation.

Our results suggest that DGKϵ is in membrane domains in which PIP2 is hydrolyzed to DAG and is activated by phosphatidylinositol signaling. In cells there is a marked enrichment of the *sn*-2 position of phosphatidylinositols with arachidonate. Thus, diacylglycerols, such as SAG, with arachidonate in the *sn*-2 position are produced more specifically by the hydrolysis of PIP2 as a consequence of the activation of the inositol signaling. We found that PIP2 is more potent in inhibiting DGKϵ (Figure 8) than other anionic lipids (*24*). If DGKϵ is bound in a region of the membrane that is rich in PIP2, it would be inactive until the PIP2 is cleaved by phospholipase C to yield SAG. This would mean that DGKϵ would be in an inhibited state in the cell until PIP2 hydrolysis was activated by a signal transduction pathway. Furthermore, stimulation of PIP2 hydrolysis will also result in the production of IP3, leading to an increase in the intracellular concentration of Ca^{2+}. This calcium would also contribute to additional activation of DGKϵ (Figure 7), resulting in an elegant negative feedback loop to terminate the DAG signal initiated by PIP2 hydrolysis. The α-, β-, and γ-isoforms of DGK have Ca^{2+}-binding EF hands (*5*), and Ca^{2+} can promote the translocation of DGKα from the cytosol to the membrane (*32*). DGKϵ does not have any identifiable calcium-binding motifs, so it is not clear by what mechanism Ca^{2+} promotes the activity of DGKϵ.

In contrast to its effects on DGKϵ, PIP2 stimulates DGKζ (Figure 8). It has been observed that the MARCKS protein, using a highly cationic group of amino acids, can sequester PIP2 into membrane microdomains (*11*). DGKζ, a Type IV DGK, has a MARCKS homology domain, containing a similar cluster of cationic residues that may bind to PIP2, which may represent a physiological mechanism to target DGKζ to PIP2-enriched membrane microdomains. Types II and V DGKs have PH domains. These domains in some proteins can bind to phosphatidylinositols, suggesting that these DGKs may also be targeted to PIP2-enriched regions. For these isoforms of DGK, activation of the phosphatidylinositol signaling pathway will cause the hydrolysis of PIP2 and the dissociation of these enzymes from the PIP2-rich domains. We suggest that the role of those latter DGK isoforms is to maintain a low concentration of DAG in the basal state in PIP2-rich membrane domains, but that in contrast, DGKϵ is responsible for turning off the signal arising from PIP2 hydrolysis.

We have demonstrated that regulation of the activity of DGK is quite different for different isoforms. Therefore, any study of mixed DGK isoforms in vitro, or any in vivo study of changes in DGK activity, cannot be generalized unless the nature of the specific isoforms involved is known. For the two isoforms studied in this work, it appears that DGKϵ acts specifically on DAG produced by inositol cycling and substances whose levels change as a consequence of inositol cycling modulate the activity of the enzyme. In contrast, DGKζ appears to be an enzyme that is always active when bound to anionic membranes. Our data demonstrate the diversity of the DGK family and suggest that their activity

in vivo may be regulated very specifically by components of the membrane microenvironment as well as signaling molecules released upon activation of cellular receptors.

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