Modulation of Diacylglycerol Kinase θ Activity by α-Thrombin and Phospholipids[†]

Becky Tu-Sekine, Michele Ostroski, and Daniel M. Raben*

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT: Diacylglycerol kinase modulates the levels of diacylglycerol and phosphatidic acid, two critical lipid second messengers, yet little is known about the effects of cellular stimulation on the kinetic behavior of this enzyme. We examined the effects of α-thrombin and activating phospholipids on the activity and substrate affinity of a soluble diacylglycerol kinase, DGKθ. Our data demonstrate that the apparent binding parameters of DGKθ increase following thrombin stimulation, suggesting that α-thrombin antagonizes DGKθ activity. Interestingly, this effect is obscured in the presence of high bulk substrate concentrations. Given the known stimulatory effects of phosphatidylserine on many diacylglycerol kinases, we examined the effects of various phospholipids on DGKθ and found that phosphatidic acid is a more effective activator than phosphatidylserine. Phosphatidic acid decreased the apparent surface K_M ($K_{M(surf)}^{app}$) of DGKθ for dioleoylglycerol (DOG) and promoted binding to vesicles in a dose-dependent manner. Phosphatidylserine also lowered the $K_{M(surf)}^{app}$ of DGKθ, though higher concentrations were required to achieve the same effect. Interestingly, PS promoted binding to vesicles only when present at levels beyond that required to saturate enzyme activity, suggesting that PS and PA activate DGKθ through different mechanisms. The potential physiological implications of these findings are discussed.

The mammalian diacylglycerol kinase (DGK)[1] family currently consists of ten isoforms (*1*, *2*). At least eight of these isoforms are freely soluble enzymes that translocate to the membranes of cells where they catalyze the ATP-dependent phosphorylation of diacylglycerol (DAG) to phosphatidic acid (PA) through an as yet unidentified mechanism. The current notion is that this lipid kinase family plays a critical role in modulating DAG levels that are increased in response to cellular stimulation (*3*−*6*). While DAG is best known as an activator of protein kinase C (PKC) family members, the identification of additional DAG binding proteins expands the potential regulatory roles of DGKs. New DAG effectors include a nuclear PIP kinase (*7*), regulators of small molecular weight G-proteins such as Ras, and a protein involved in exocytosis (Munc13). These additional DAG binding proteins have highlighted the vital signaling role of this lipid (*8*).

In light of the fact that DGK is poised to simultaneously regulate the levels of two critical lipid second messengers, diacylglycerol (DAG) and phosphatidic acid (PA), surprisingly little is known about the regulation and mechanisms of activation of the DGK family of enzymes. To begin to address the question of DGK activation, we have examined the effects of α-thrombin stimulation and exposure to common phospholipids on the kinetic behavior of DGKθ. α-Thrombin promotes a variety of responses in different cell types, and DGKθ has been shown to translocate to subcellular membranes and compartments in response to thrombin stimulation (*9*, *10*), but the effect of this stimulation on the intrinsic activity of DGKθ has not been studied. To better understand the mechanism of DGK activation, we have examined the effects of α-thrombin stimulation and exposure to activating phospholipids on the kinetic behavior of DGKθ using an approach similar to that of Epand and co-workers, in which the potential influences of endogenous protein interactions on DGKα or DGKζ binding and activity were preserved by utilizing cytosol containing overexpressed enzyme. While most studies report that phospholipid activators increase interfacial binding, the Epand study reported an increase in activity that was disproportional to the increase in enzyme binding, suggesting that phospholipids may also influence substrate binding or the enzyme turnover rate (*11*). This prompted us to study the effect of phospholipids on the kinetics of DGKθ present in cytosol from quiescent and α-thrombin-induced cells.

Interfacial Enzymes: General Considerations. Kinetic Parameters $K_{M(surf)}$ and K_M^{app}. Interfacial enzymes such as DGK are distinctive in that they metabolize substrate at a lipid/aqueous interface. While the enzyme itself may be soluble (as with DGKθ) and can, therefore, move freely between cellular compartments, the DAG substrate is insoluble and confined to a membrane structure or amphipathic aggregate. This difference in solubility necessitates an interfacial binding step before the enzyme can interact with this substrate. Because the enzyme is then confined to a two-dimensional surface, the substrate affinity (the $K_{M(surf)}$) is presented in terms of the mole fraction of total lipid, rather

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* Address correspondence to this author. Tel: 410-955-1289. Fax: 410-614-8729. E-mail: draben@jhmi.edu.

[1] Abbreviations: DAG, diacylglycerol; DOG, 1,2-dioleoyl-*sn*-glycerol; diC8, 1,2-dioctanoyl-*sn*-glycerol; DGK, diacylglycerol kinase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PAP, phosphatidic acid phosphohydrolase; PKC, protein kinase C; PLD, phospholipase D.

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than in bulk concentration terms (*12*). [For consistency, we have used the symbols for kinetic variables as presented by Berg and Jain (*13*).]

Enzymes that do not bind tightly to the interface complicate kinetic analyses because the repeated binding (interfacial binding affinity) influences the overall reaction rate. To account for this "hopping" behavior, Berg and Jain derived a new binding equilibrium, the K_M^{app} (*13*). This parameter reflects the combined effects of the interfacial binding step and substrate binding. The data in this report and elsewhere (*14*, *15*) indicate that a number of the DGKs are hopping interfacial enzymes. Therefore, we have employed the methods described by Berg and Jain in our kinetic interpretation.

In the present study, we examined the kinetics of DGKθ in both vesicles and octyl glucoside mixed micelles, a detergent system routinely used in the assay of this enzyme family. Use of a micellar system was advantageous because there is rapid exchange of substrate and product between micelles, primarily via collisions (*16*). This occurs at a rate that is 3−4-fold greater than the highest measured turnover rate for a DGK (*17*) and 20-fold greater than the turnover rate we recently determined for the DGKθ homologue, DGKA (*18*). This fast exchange ensured that the surface concentrations of product and substrate remained constant throughout the population of micelles, particularly under the conditions of initial velocity used for this study. Using these systems, we determined that thrombin stimulation resulted in an increase in the K_M^{app} of DGKθ at low concentrations of substrate (DOG) and that increasing the bulk DOG concentration returned the K_M^{app} to the basal value. Our data suggest that α-thrombin stimulation antagonizes DGKθ activity under conditions where DAG bulk concentrations are low and that this effect is obscured in the presence of high bulk substrate concentrations. In addition, we observed that phosphatidic acid is a more efficient activator of DGKθ than PS under the conditions examined and that these phospholipids appear to activate DGKθ by different mechanisms.

EXPERIMENTAL PROCEDURES

Materials. Silica gel 60 TLC plates (aluminum sheets) were purchased from EM Science (Germany). Cytoscint scintillation-counting fluid was obtained from ICN (Costa Mesa, CA). Tissue culture media components were purchased from MediaTech, Inc. (Herndon, VA). Plastic culture dishes were purchased from Falcon Labware. Highly purified human thrombin (~4000 NIH units/mL) and BSA (RIA grade, fraction V) were purchased from Sigma (St. Louis, MO). Octyl β-glucoside was from Calbiochem (Santa Cruz, CA). All lipids were purchased from Avanti. Novafector was purchased from Venn Nova (Pompano Beach, FL). Other chemicals were of reagent grade.

Cells and Cell Culture. IIC9 cells, a subclone of Chinese hamster embryo fibroblasts (CHEF19, ATCC), were grown, maintained, and serum starved as previously described (*19*), with the exception that 7.5% FBS was used on growing cells. Cultures were serum-deprived by washing with low-glucose Dulbecco's modified Eagle's medium (D-MEM) and incubating in fresh D-MEM containing 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine for 40−48 h. The cultures were then incubated in fresh low-glucose D-MEM in the presence or absence of α-thrombin (1.5 NIH units/mL) for the indicated times.

Transient Transfections. IIC9 cells were grown in 175 cm^2 flasks until 50−60% confluent. Cultures were washed once with PBS and once with OPTIMEM I (reduced serum medium of Eagles's MEM; Life Technologies) and then incubated in 10 mL of OPTIMEM I containing 20 μg of cDNA in 100 μL of Novafector. Following an overnight incubation, 10 mL of 15% FCS-containing complete medium with 2× penicillin, streptomycin, and L-glutamine was added, and cells were grown for an additional 12−18 h before being subjected to serum deprivation. Transfection efficiency was evaluated by parallel transfections with 20 μg of pEGFP-N3 vector (Clontech).

Constructs. hDGKθ cDNA inserted in pcDNA3 vector was kindly provided by Dr. W. J. van Blitterswijk (Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam).

Preparation of Cellular Fractions. Cytoplasmic fractions were prepared as follows: cells were washed 2 × 20 mL in ice-cold FB (10 mM Hepes, pH 7.4, 1 mM EDTA, 0.5 mM EGTA) and allowed to swell on ice for 10−20 min in ice-cold FB with inhibitors (1 mM AEBSF, 10 μg/mL aprotinin, 10 μM leupeptin, 20 μM quinacrine, 10 mM NaF, 1 mM $NaVO_3$, pepstatin A, 1× Sigma phosphatase inhibitor mix I). Cells were scraped and dounced 25−35 times with a type B pestle (Konte Glass, Vineland, NJ). Nuclei and unbroken cells were removed by centrifugation at 400*g*, 4 °C, for 15 min. Membranes were removed from the resulting suspension by ultracentrifugation at 100000*g* for 1 h at 4 °C. Membrane-free cytosol was supplemented with 13 mM $MgCl_2$ and 0.1% Tx100 and aliquoted prior to storage at −80 °C. An aliquot was thawed immediately before use, and unused portions were discarded.

DGK Micellar Assays. DGK activity was assayed using octyl glucoside/diacylglyceride (OG/DAG) mixed micelles as previously described (*9*). Manufacturer (Calbiochem) values for the CMC (25 mM) and aggregation number (*88*) of OG were used to calculate the concentration of micellar OG and the micelle number. In brief, lipids stored in chloroform were aliquoted, dried under nitrogen, and stored under vacuum at 4 °C for at least 1 h to remove residual $CHCl_3$ prior to forming micelles. Dried lipids were resuspended in an OG buffer solution by vortexing and sonicating until the suspension was clear (approximately 2−5 min). The reaction was started by addition of 90 μL of OG/[^{32}P]ATP reaction mix to 10 μL of protein (approximately 2−5 μg of protein) on wet ice, and the tubes were gently shaken prior to incubation for 15 min in a 30 °C water bath. Final assay conditions were 50 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 10 mM NaF, 1 mM dithiothreitol, and 1 mM [γ-^{32}P]ATP with a specific activity of 2.5 × 10^5 cpm/nmol. DTT and [^{32}P]-ATP were added to the micelles just prior to the reaction. Reactions were terminated by the addition of chloroform/methanol/1% perchloric acid (1:2:0.75) (v/v), and phases were separated by addition of 1 mL each of $CHCl_3$ and 1% perchloric acid. The organic phase was washed with 2 mL of 1% perchloric acid, then dried under nitrogen gas, and spotted onto a silica gel 60 TLC plate. Phosphatidic acid (PA) was separated from other lipids using a solvent system containing chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1) (v/v). The amount of [γ-^{32}P]PA was measured

by liquid scintillation spectrophotometry in a Wallac 1410 liquid scintillation counter. DGKθ activity was quantified as nanomoles of PA per microgram of total cytosolic protein. All assays were linear with respect to time and protein concentration (data not shown). When used, propranolol was added at a final concentration of 1.5 mM to the reaction mix.

DGK Vesicle Assays. Lipids stored in chloroform were aliquoted, dried under nitrogen, and stored under vacuum at 4 °C for at least 2 h to remove residual $CHCl_3$ prior to forming vesicles. Dried lipids were resuspended in 55 mM Tris-HCl, pH 8.2, containing 5.5 mM $MgCl_2$ and 10 mM NaF. Lipids were rehydrated for 30 min at 45 °C and then freeze-thawed five times on dry ice prior to 11 passes through a 0.1 μm polycarbonate membrane using an Avanti miniextruder. The base lipid composition of vesicles consisted of PE and PC in a ratio of 2:1 (mol %). The PE:PC ratio was maintained in all vesicle compositions utilized in this study.

DGK Binding Assays. The binding assay was conducted as described by Thomas et al. (*14*) with some modifications. Vesicles were prepared as described above in buffer containing 176 mM sucrose and recovered by centrifugation at 20 °C for 20 min at 80000 × *g*. The supernatant was removed, and pellets were resuspended in DGK assay buffer supplemented with 1 mM ATP and 1 mM DTT. Protein was added to the vesicle solution (final volume 100 μL) and incubated at room temperature for 10 min; then vesicles were recovered by centrifugation for 20 min at 80000 × *g*, 20 °C. The supernatant was removed and saved for an activity assay. The pellet was washed briefly with assay buffer, resuspended in 100 μL of 1% Tx114, and incubated on ice for 5 min to solubilize the vesicles. Samples were incubated at 30 °C for 3 min to precipitate the detergent and spun at 14 000 rpm in a microcentrifuge for 5 min to pellet the lipid/detergent complexes. An aliquot of the supernatant was assayed for DGK activity using 10 mM Tx100 mixed micelles containing 6 mol % DOG and 9 mol % PS.

RESULTS

DGKs are activated in a number of systems by a variety of agonists. One important question that has emerged from these studies is whether the observed "activation" of DGK is associated with alteration of a kinetic parameter or whether the apparent activation is due solely to a change in the local concentration of enzyme that results from cellular redistribution. Addressing this question requires an investigation of the apparent kinetic parameters and the effect of agonist stimulation on these parameters. Similar to other studies that showed an increase in DGKθ activity in cellular membranes following agonist stimulation (*20*), work from our laboratory demonstrates that stimulation of fibroblasts with α-thrombin leads to an increase in DGKθ activity in nuclei and membranes (ref *9* and data not shown). Therefore, we chose to examine DGKθ in this system and to determine the effect of α-thrombin and known phospholipid activators on the kinetic behavior of this enzyme. For this initial study, we examined the cytosolic form of the enzyme to avoid complications in the analysis due to the presence of membrane phospholipids.

DGKθ was overexpressed in IIC9 fibroblasts, and the total DGK activity levels were measured and compared to control,

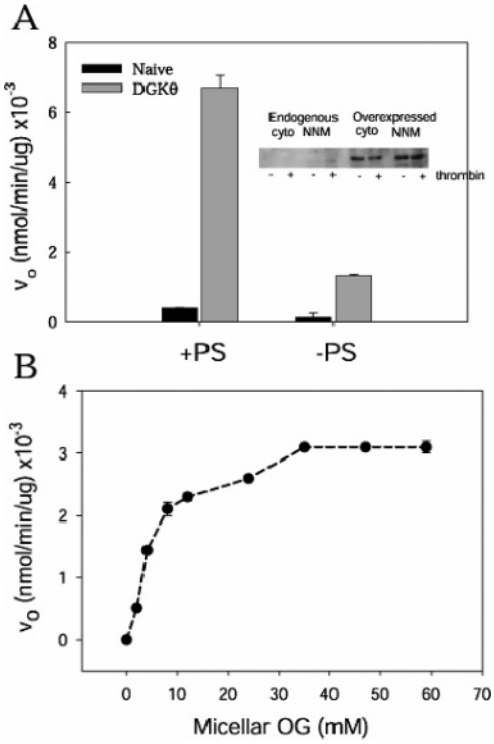

FIGURE 1: (A) hDGKθ is the primary DGK activity in transfected fibroblasts. Cells transfected with GFP (naive) or hDGKθ were brought to quiescence and fractionated. Equal amounts of membrane-free cytosol were assayed for DGK activity in OG/DOG mixed micelles in the presence or absence of PS as described. Black bars = naive; gray bars = hDGKθ. Inset: Western blot of DGKθ in cytosol (cyto) and nonnuclear membranes (NNM) in naive cells (endogenous) and cells overexpressing DGKθ. (B) OG does not inhibit hDGKθ activity. Prepared cytosol was assayed for DGK activity at increasing concentrations of OG micelles containing 6 mol % DOG and 8.1 mol % PS. OG concentration is represented as mM micellar OG, not as total detergent concentration. Results are representative of two experiments completed in duplicate.

naive cells expressing GFP alone. Importantly, DGK activity in cytosol isolated from cells overexpressing DGKθ was consistently 10-fold greater than in GFP-transfected controls (Figure 1A), indicating that the other two DGK isoforms present in these cells (DGKδ and DGKζ) (*9*) did not contribute significantly to the measured DGK activity. To verify that DGKθ activity was not affected by the detergent (OG) micelles, we examined DGK activity over the range of OG micellar concentrations used in these studies. The surface concentrations of substrate (DOG) and PS were held constant (6 and 8 mol %, respectively), and the OG micellar concentration was varied. We found that DGK activity saturated between 5 and 10 mM OG micelles under these conditions and that increasing the levels of OG did not result in a loss of DGK activity over the range of concentrations tested (Figure 1B). This result indicates that OG does not adversely impact the enzyme under these conditions. In addition, we compared expression levels of DGKθ by IP and found that stimulated cytosol contained slightly less DGKθ than quiescent cytosol, which corresponded with a minor increase in DGKθ on membranes following thrombin stimulation (data not shown).

DGKθ Activity Is Dependent on both the Surface and Bulk Concentrations of Substrate (DOG). An identifying characteristic of interfacial enzymes is their sensitivity to changes in the surface concentration of substrate. Therefore, we

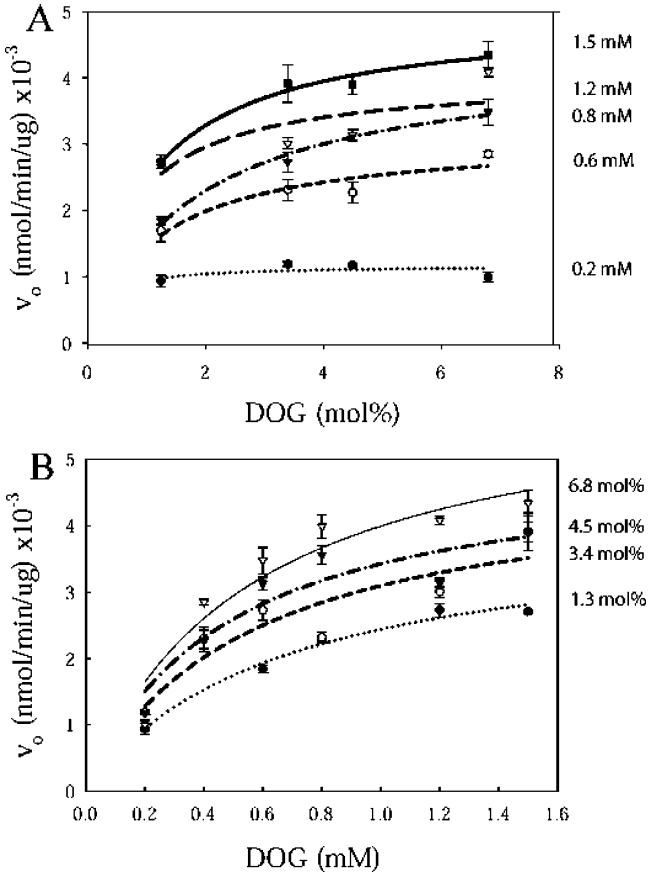

FIGURE 2: DGKθ is dependent on both the surface and bulk concentration of DOG. Cytosol from cells expressing DGKθ was assayed over a range of DOG concentrations (0.2−1.5 mM) at constant mol % (1.25, 3.4, 4.5, and 6.8) in OG mixed micelles containing 8.1 mol % PS and plotted with respect to dependence on (A) surface concentration of DOG and (B) bulk concentration of DOG. Results are representative of two experiments completed in triplicate. Error bars indicate standard deviation.

assayed DGKθ activity in membrane-free cytosol over a range of substrate surface concentrations (1.25, 3.4, 4.5, 6.8 mol %) at fixed bulk substrate concentrations (0.2−1.5 mM) in the presence of 8 mol % PS. Under these conditions, DGKθ activity increased with increasing substrate surface concentration, confirming that DGKθ is an interfacial enzyme (Figure 2A). The fact that DGKθ is dependent on both the surface (Figure 2A) and bulk (Figures 1B and 2B) concentrations of substrate implies that DGKθ is not a highly processive enzyme, i.e., that it is a "hopper". We observed the same hopping behavior from membrane-bound DGKθ (Supporting Information, Figure S2), indicating that this characteristic does not change following membrane association. We should note that when the bulk substrate concentration was maintained at 0.2 mM, there was no detectable dependence on surface concentration (Figure 2A), suggesting that bulk substrate concentrations greater than 0.2 mM are likely to be important for micelle binding under these conditions.

PA Is a More Efficient Activator of DGKθ Than PS. PS is a well-known phospholipid activator of many DGKs in *in vitro* assays. The mechanism involved in this activation is not entirely clear. While PS-induced activation of various DGK isoforms occurs in a concentration-dependent manner, the amount of PS required and the dependence on other cofactors (such as Ca^{2+} in the case of DGKα) vary between

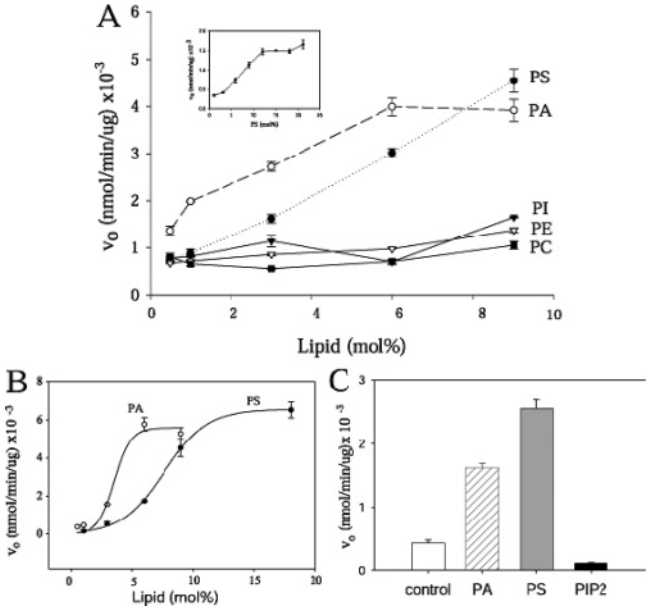

FIGURE 3: DGKθ is activated by PA and PS. Membrane-free cytosol from thrombin-stimulated cells expressing DGKθ was assayed (A) in 15 mM octylglucoside mixed micelles using 3 mol % DOG over a range of surface concentrations of five common phospholipids (open circles = PA; closed circles = PS; closed triangles = PI; open triangles = PE; closed squares = PC), (B) in 15 mM small unilamellar vesicles (SUVs) containing 3 mol % DOG over the indicated range of PS or PA (open circles = PA; closed circles = PS), and (C) in 2.5 mM SUVs containing 4 mol % DOG and 6 mol % PA, 9 mol % PS, or 2 mol % PIP_2. Hatched bars = PA; gray bars = PS; black bar = PI(4,5)P_2; white bar = control. Results are representative of two experiments completed in triplicate. Error bars represent standard error. Inset: DGKθ activity was assayed over an increasing range of PS surface concentrations to saturate activity for determination of the K_{act}.

isoforms (*21*−*24*). In addition, other phospholipids have been shown to affect DGK activity. For example, we recently reported that PA is a potent activator of DGKA, a homologue of DGKθ found in *Dictyostelium discoideum* (*18*). Thirugnanam et al. reported that PA and phosphatidylinositol (PI) activate DGKζ and proposed that anionic lipids activate this isoform in a charge-dependent manner (*25*). In addition, DGKθ contains a pleckstrin homology (PH) domain and has been colocalized to regions rich in PIP_2 (*26*). These reports prompted us to evaluate the effects of PS, PA, PI, and PIP_2 on the kinetics of DGKθ. To investigate the possibility that DGKθ is activated by phospholipids in general, we also tested two neutral lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Due to solubility limitations in OG micelles, the effect of PIP_2 on activity was determined in phospholipid vesicles only.

DGKθ activity was unaffected by PI, PC, and PE in OG micelles. PA activated DGKθ at surface concentrations as low as 1 mol % while PS was a poor activator below 3 mol % (Figure 3A). In OG mixed micelles, the K_{act} for PA was 2.5 (±2.5) mol % while the K_{act} for PS was 10 (±3.0) mol %. In unilamellar vesicles (Figure 3B), PA also activated DGKθ more efficiently, with a K_{act} of 3.5 ± 0.1 mol %, while PS exhibited a K_{act} of 6.6 ± 1.0 mol %. PIP_2 had no apparent effect on activity (Figure 3C).

PA and PS Differentially Influence DGKθ Activity. Next, we examined the influence of the activating lipids on the vesicle binding and K_M^{app} of DGKθ. In order to compare

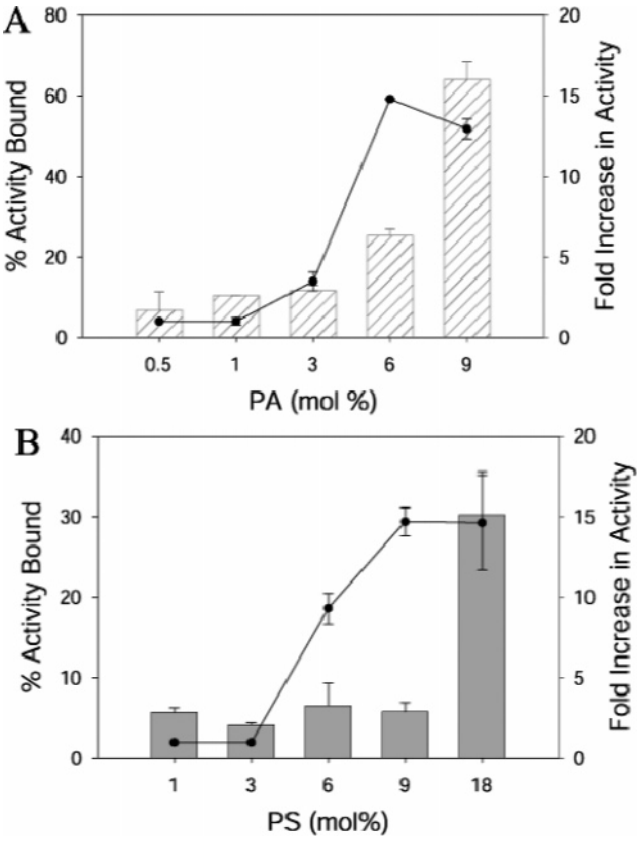

FIGURE 4: PA and PS exert differential effects on binding of DGKθ to vesicles. Membrane-free cytosol from thrombin-stimulated cells was assayed for DGKθ activity and binding using SUVs as described in Experimental Procedures at the indicated ranges of (A) PA and (B) PS concentrations. Fold change in specific activity is depicted as a line graph, and % bound activity is presented as a bar graph (hatched bars = PA; gray bars = PS). Data are representative of two experiments conducted in triplicate (activity) and quadruplicate (binding).

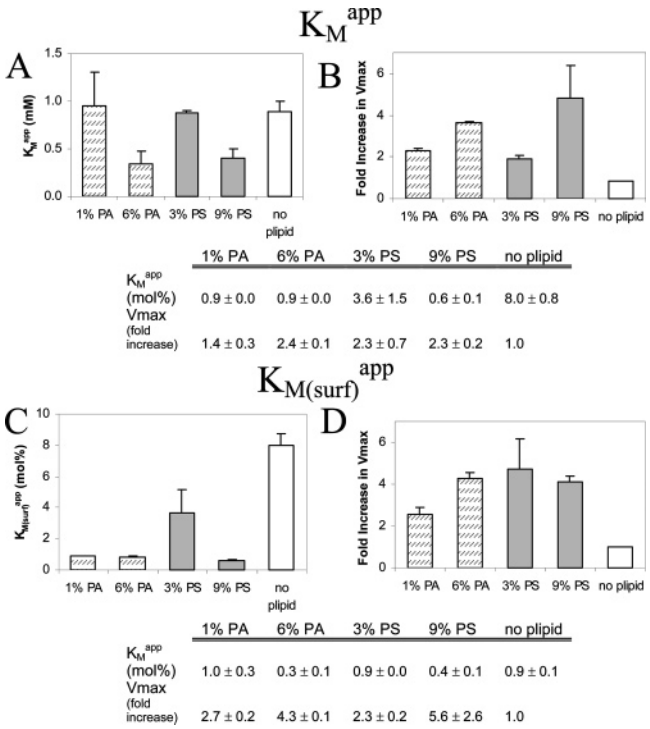

FIGURE 5: PA and PS alter the K_M and V_{max} values of DGKθ. Membrane-free cytosol from thrombin-stimulated cells expressing DGKθ was assayed in the presence of PA or PS to determine the effect on (A) the K_M^{app} and (C) the $K_{M(surf)}^{app}$. The surface concentration of DOG was fixed at 3 mol %, and bulk DOG was varied from 0.08 to 1.35 mM in (A) while the bulk concentration of DOG was held constant at 0.8 mM and the surface concentration of DOG was varied from 0.5 to 9.0 mol % in (C). The fold increase in V_{max} values relative to no phospholipid controls was determined for each experiment (B, D). Results are the mean of two or three experiments completed in triplicate. All values are presented as tabulated results beneath the graphs. Error bars represent standard error. Hatched bars = PA; gray bars = PS; white bars = control without PA or PS.

the effects of the two lipids, we chose surface concentrations for PA (1 and 6 mol %) and PS (3 and 9 mol %) that produced similar levels of DGKθ activity (see Figure 3A). Binding assays were conducted using sucrose-loaded vesicles as described in Experimental Procedures to determine the effect of PA and PS on binding. The data indicate that both phospholipids increased activity prior to detectable increases in binding. PA differed from PS in that the amount of bound enzyme increased in a linear fashion in response to increases in PA (Figure 4A), while no detectable increase in enzyme binding to PS-containing vesicles was observed until the PS concentration reached 18 mol % (Figure 4B). Interestingly, enzyme binding did not saturate over the range of PA and PS tested, while activity saturated at 6 mol % PA and 9 mol % PS.

We next evaluated the effect of low and high levels of PA and PS on the K_M^{app}, $K_{M(surf)}^{app}$, and V_{max} parameters. To determine the K_M^{app}, we held the surface concentration of substrate (DOG) constant at 3 mol % and varied the bulk substrate concentration from 0.075 to 1.35 mM. The K_M^{app} for DOG appeared to be unchanged over the no phospholipid control in the presence of low levels of PA (1 mol %) and PS (3 mol %). However, when the concentrations of PA and PS were increased, the K_M^{app} dropped by approximately 3-fold in both cases. At the maximum concentrations of phospholipid tested, the K_M^{app} in the presence of PA was 0.3 (±0.1) mM DOG, and in the presence of PS the K_M^{app} was 0.4 (±0.1) mM DOG. The effect on the V_{max} was similar for the two phospholipids, with low levels of PA or PS producing an approximately 2-fold increase in the V_{max} over controls, while higher levels resulted in an approximately 3-fold increase (Figure 5A,B).

We investigated the effect of these phospholipids on the $K_{M(surf)}^{app}$ by varying the surface concentration of DOG at a constant bulk concentration of DOG (0.8 mM). The addition of PA decreased the $K_{M(surf)}^{app}$ over the control at the lowest concentration tested (1 mol % PA), and increasing the concentration of PA did not further affect the $K_{M(surf)}^{app}$ for DOG, which remained constant at 0.9 ± 0.09 mol %. However, we observed a dose-dependent decrease in the $K_{M(surf)}^{app}$ in the presence of PS. A low level of PS (3 mol %) dropped the $K_{M(surf)}^{app}$ for DOG approximately 2-fold, while 9 mol % PS produced a 6-fold drop in the $K_{M(surf)}^{app}$ (0.6 ± 0.09 mol %) relative to controls (Figure 5C). Consistent with the binding data, we observed a 2-fold increase in V_{max} when PA was increased from 1 to 6 mol % but observed no change in the V_{max} when PS was increased from 3 to 9 mol % (Figure 5D). These data support the notion that the two lipids activate DGK by different mechanisms, with PA acting as a recruiting molecule while PS primarily influences the surface kinetics of DGKθ.

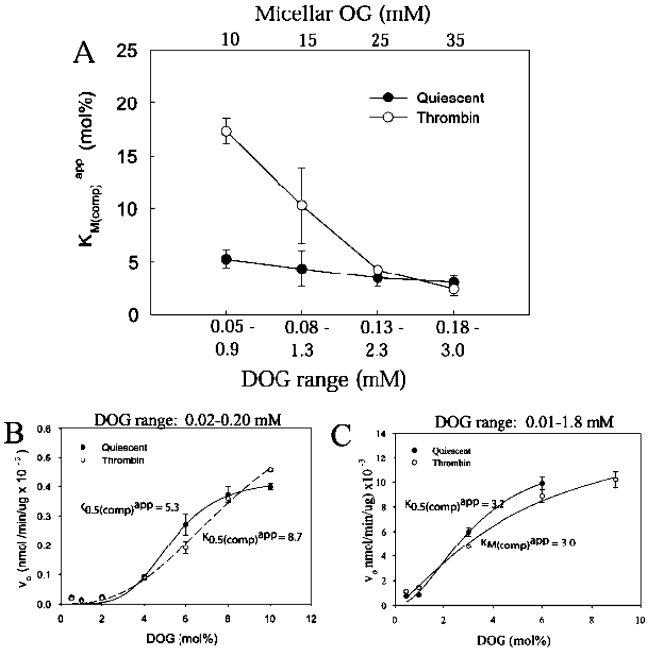

FIGURE 6: Thrombin stimulation alters the $K_{M(comp)}^{app}$ in a bulk substrate-dependent manner. The $K_{M(comp)}^{app}$ of cytosolic DGKθ from quiescent or thrombin-stimulated cells was determined over a set range of DOG surface concentrations (0.5, 1.0, 3.0, 6.0, and 9.0 mol % DOG) at increasing bulk DOG concentrations. Assays were done in the presence of 8.1 mol % PS. Data shown are averages of two (quiescent) or three (thrombin) experiments completed in triplicate using cytosol from different cell harvests. Each data point represents the $K_{M(comp)}^{app}$ from a single experiment completed in triplicate. Standard error was typically less than 5% in each experiment. The $K_{M(comp)}^{app}$ was also determined in SUVs at (B) low DOG and (C) high DOG concentrations. The data are representative of two experiments completed in triplicate. Open circles = quiescent DGKθ; closed circles = thrombin-stimulated DGKθ.

Thrombin Alters the Binding Parameters of DGKθ. An important question that emerged in this study was whether thrombin stimulation of quiescent fibroblasts altered the kinetic behavior of DGKθ. In order to answer this question, it was important to mimic physiological conditions in which the total amount of interface remains relatively constant while the local bulk and surface concentrations of lipids change simultaneously. To accomplish this, we evaluated the effect of thrombin on DGKθ under conditions of constant interface while bulk and surface concentrations of the substrate varied. For clarity, we refer to the K_M determined by this approach as a "composite K_M^{app}" ($K_{M(comp)}^{app}$) since it is influenced by both the surface and bulk substrate concentrations. In this study the $K_{M(comp)}^{app}$ was quantified over a constant range of substrate surface concentrations (0.5−9 mol %). To evaluate the effects of bulk substrate on the kinetics, we quantified the $K_{M(comp)}^{app}$ at increasing ranges of bulk DOG concentration. Interestingly, the data showed that the $K_{M(comp)}^{app}$ of α-thrombin-stimulated DGKθ was substantially higher than that of quiescent DGKθ when assayed at low bulk substrate concentrations. Further, the $K_{M(comp)}^{app}$ values for both samples converged as the bulk concentration of substrate increased (Figure 6A). Similar results were observed in assays conducted in the absence of PS (data not shown). We repeated this experiment using a saturating concentration of lipid vesicles and again found that the $K_{M(comp)}^{app}$ for thrombin-stimulated DGKθ was greater than that of quiescent DGKθ at low bulk DOG concentrations but converged toward a common value at higher bulk DOG concentrations (Figure 6B,C).

DISCUSSION

In the present study we have examined the kinetic behavior of DGKθ in membrane-free cytosol to evaluate the effects of potential physiological activators including stimulation by α-thrombin and specific cellular phospholipids. Our studies show that α-thrombin, phosphatidylserine, and phosphatidic acid alter the kinetic behavior of DGKθ in this system.

Differential Activation of DGKθ by PA and PS. Anionic phospholipids have been shown to activate or inhibit DGKs in an isoform-specific manner. Our examination of the effects of PS and PA on the kinetics of DGKθ showed that they activate DGKθ in a similar, but not identical, fashion. For example, PA activated DGKθ at 6-fold lower surface and bulk concentrations than PS. Significantly, the surface concentrations of lipid that were required to produce notable changes in the K_M and V_{max} values were at physiological levels for both lipids. In IIC9 fibroblasts, for example, PS comprises 16%, and PA comprises 3%, of total lipid (*27*). While it is almost certain that only a portion of the cellular PA pool is available to DGK, local PA levels resulting from DGK or PLD activity could reasonably exceed 1 mol %.

Surprisingly, our studies revealed that PS stimulated activity without a detectable increase in bound enzyme (Figure 4B). A similar observation on the apparent incongruency between binding and activity was made by Epand and co-workers, who noted that increases in DGKα and DGKζ activity appeared to exceed measured increases in binding (*11*), suggesting that the intrinsic activity of the enzyme was altered. We did detect an increase in vesicle-bound enzyme in the presence of PA, with 6 mol % PA producing a 2-fold increase in bound enzyme; however, the measured 2-fold increase in enzyme correlated with an approximately 8-fold increase in activity, consistent with a change in intrinsic activity.

Similar binding effects for PS and PA were observed in an elegant study conducted by Thomas and Glomsett using a partially purified DGK isolated from the cytosol of NIH 3T3 cells. This study reported that the K_d for PA vesicles was lower than the K_d for vesicles which contained twice the amount of PS and that PS binding was poor below 25 mol % (*14*). The effect of PA on binding is especially interesting as it tempts the suggestion that DGKθ is positively regulated by its product or that PLD activity may regulate DGKθ activity.

Low levels of PA or PS altered the $K_{M(surf)}^{app}$ without noticeably affecting binding to vesicles (see Figures 4 and 5), suggesting that at least part of the activating effect of these phospholipids is through a change in the substrate affinity of the enzyme. However, their effects on interfacial binding are not the same. PA increased binding of DGKθ to vesicles by 2-fold when the concentration of PA was increased from 1 to 6 mol %, consistent with the measured 2-fold increase in V_{max}. Like PA, PS lowered the K_M values, yet no commensurate increase in binding was observed. This is similar to the effects of PA and PS on a related lipid kinase, sphingosine kinase 1, as measured by surface plasmon resonance (*28*). In these studies, PA increased the rate of

vesicle association approximately 2-fold, while PS had no significant effect on this rate constant.

We find it curious that high levels of either lipid promoted binding without further increasing enzyme activity (we should note here that substrate was always in great excess in all of the experiments conducted, so activity could not have saturated due to substrate depletion). This suggests that the increase in binding was nonproductive since the additional enzyme could not process substrate. The reason for this is unclear and requires further study.

It is interesting to note the PIP_2 did not activate the enzyme, even though DGKθ contains a putative PH domain. However, it is also possible that this PH domain binds PIP_3 or promotes binding to a protein partner rather than a lipid (*29*). An alternative explanation is that PIP_2 and/or PIP_3 promote binding of DGKθ to the interface only in combination with another activating lipid, such as PS or PA, or that they promote binding in such a way as to inactivate the enzyme, possibly by orienting the active site away from the interface. Further study will be necessary to determine the role, if any, of the phosphatidylinositols in activation or binding of this enzyme.

Thrombin Stimulation Influences DGKθ Interfacial Binding. One significant finding resulting from this study is that thrombin alters the enzyme's sensitivity to DOG at concentrations below the K_M^{app}, suggesting that thrombin stimulation attenuates enzyme activity. While the data we have presented arise from study of soluble DGKθ in membrane-denuded cytosol, it is important to remember that membrane-associated DGKθ, like the cytosolic form, is a hopping enzyme (Supporting Information, Figure S2). Therefore, it is not unreasonable to conclude that there is a dynamic exchange of enzyme between the cytosolic and membrane-bound pools of the enzyme and that the observed changes in the kinetic behaviors of the cytosolic enzyme represent the membrane-bound enzyme to some degree. This observed increase in the K_M seemed counterintuitive since we have previously observed that thrombin stimulation causes DGKθ to translocate both to the nucleus and to membranes (ref *9* and data not shown) and that in vitro activity measurements of these stimulated fractions are correspondingly greater than unstimulated controls. However, this apparent antagonism of DGKθ is more easily understood in the context of the overall thrombin-induced signaling network. It is clear from our study and others that most DGK isoforms are constitutively active [DGKδ has been found to exist as an inactive multimer in the cytosol (*30*)], suggesting that they function as housekeeping enzymes to help to regulate the basal level of DAG. In this view, it is likely that an immediate increase in DGK activity following stimulation by an agonist would result in a rapid, possibly premature, abrogation of the newly initiated DAG signal. However, an increase in the K_M for DAG, like that observed in our study, would serve to attenuate enzyme activity until DAG levels exceeded the new substrate affinity threshold or until a subsequent change restored the original K_M value. While the molecular mechanism for this regulation cannot be inferred from this study, the role of DAG may be to act as an allosteric activator, perhaps by engaging the C1 domains.

The observed effect of thrombin on the $K_{M(comp)}^{app}$ of DGKθ is consistent with a conformational change of the enzyme after stimulation, perhaps through a simple post-translational modification of the enzyme such as phosphorylation. Indeed, DGKθ was recently reported to undergo phosphorylation by PKCϵ in COS7 cells that had been treated with extracellular ATP, bradykinin, or thrombin (*10*). α-Thrombin has been shown to activate PKCϵ in IIC9 cytosol (*31*), though we have not yet investigated a PKCϵ-dependent phosphorylation of DGKθ following thrombin stimulation.

Alternatively, a protein partner may be responsible for the increase in the $K_{M(comp)}^{app}$. For example, it has been shown that GTP-bound RhoA binds to and inhibits DGKθ (*32*). However, we feel that RhoA·GTP is unlikely to cause this change in the K_M since it has been shown to bind to the catalytic site and abrogate DGKθ activity, which would manifest as a change in the V_{max}.

We should also call attention to the possibility that the observed thrombin effect could result from degradation of PA by a thrombin-stimulated PA phosphohydrolase present in the cytosol. While most lipid-metabolizing enzymes are associated with the membrane, and therefore are unlikely to interfere with the measured DGK reaction, a soluble form of phosphatidic acid phosphohydrolase (PAP) has been identified in mammalian cells (*33*). However, we found that addition of 1.5 mM propranolol did not selectively alter the kinetics of the thrombin-induced form of the enzyme but rather increased the $K_{M(comp)}^{app}$ of both the quiescent and stimulated forms of DGKθ by approximately 10−20%, indicating that the observed thrombin effect is not due to a propranolol-sensitive activity (data not shown).

A Working Model for the in Vivo Function and Regulation of DGKθ. The effect of thrombin and phospholipid activators on DGKθ suggests that this enzyme performs a dual function in IIC9 fibroblasts. We have constructed a preliminary model proposing that DGKθ functions as a housekeeping enzyme to maintain basal levels of DAG until the cell is activated (e.g., by α-thrombin), while in stimulated cells DGKθ terminates DAG-dependent signaling cascades once the local DAG levels exceed a threshold level. One interpretation of the increase in the $K_{M(comp)}$ is that DGKθ enzyme is "desensitized" following stimulation. This attenuation would prevent DGKθ from disturbing local increases in DAG necessary to activate downstream signaling effectors and may increase DGKθ activity at regions containing high levels of DAG to terminate these signals. In addition, the observed stimulation of DGKθ activity by PS supports the notion that DGKθ acts as an "off switch" for PKCα, since regions rich in PS would activate DGKθ. Our data also suggest that PS can act as a recruiting mechanism when it is present at relatively high concentrations (over 9 mol %), further strengthening the potential relationship between DGKθ and PKCα.

One obvious mechanism of DAG attenuation is through a thrombin-induced change in the structure of the C1 domains. All DGKs contain regions that exhibit some homology to C1 domains of PKC and were initially predicted to bind DAG. However, only the DGKγ and DGKβ C1 domains are homologous with the PKC C1 core sequence, and to date only these isoforms have been clearly shown to bind phorbol ester with high affinity (*34*). Until recently, the function of the C1 domains for the other DGKs has been unclear. However, binding and in vivo studies on two soluble DGKs have clearly shown that these enzymes bind DAG both in

vitro and in vivo. Glomsett and co-workers showed that a soluble DGK from Swiss 3T3 cells binds 100-fold more strongly to vesicles in the presence of DAG (*14*). In addition, recent work by van Baal et al. has shown that DGKθ can be induced to translocate to the plasma membrane following the addition of diC8 DAG, a medium-chain DAG, and that this response is eliminated by mutation of the C1 domains (*10*). Our data are consistent with the findings of others that show C1 domains have moderate affinity for DAG and suggest that these domains are important for DGK regulation.

In this study we have shown that DGKθ is dependent on both the surface and bulk concentration of substrate and belongs to the class of interfacial enzymes known as "hoppers". Significantly, binding and activity studies showed that PS activates DGKθ by increasing substrate affinity in the absence of increased binding, while PA increases substrate affinity and recruits enzyme to the interface. The finding that even relatively low levels of PS decrease the $K_{M(surf)}^{app}$ of DGKθ for DOG suggests that this enzyme is a more efficient regulator of DAG levels in PS-rich membranes than in PS-poor ones and supports the prevailing notion that DGKθ is a negative regulator of PS-dependent DAG effectors such as PKCα. Further, our data show that this increase in activity occurs without a detectable increase in interfacial binding, suggesting that translocation of DGKθ to membranes does not directly reflect in vivo changes in activity.

In addition, we have shown that DGKθ activity is attenuated by thrombin stimulation, providing insight into the regulation of the enzyme in vivo. Further, this change is only observed under assay conditions of very low bulk concentrations of substrate, suggesting that the current method of assaying for DGK activation in the presence of saturating bulk substrate primarily provides information on the total mass amount of enzyme.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 containing a sample of the reaction velocity curves used to construct the graph in Figure 6A and Figure S2 containing velocity curves depicting the hopping behavior of membrane-associated DGKθ. This material is available free of charge via the Internet at http://pubs.acs.org.

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