Analysis of a Novel Diacylglycerol Kinase from *Dictyostelium discoideum*: DGKA†

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ABSTRACT: Diacylglycerol kinases (DGKs) catalyze the ATP-dependent phosphorylation of diacylglycerols to generate phosphatidic acid and have been investigated in prokaryotic and eukaryotic organisms. Recently, a protein that is significantly similar to human DGK-θ, DGKA, was identified in *Dictyostelium discoideum.* It has been shown to possess DGK activity when assayed using a medium-chain diacylglycerol, 1,2-dioctanoyl-*sn*-glycerol (DiC8). A complete understanding of DGK catalytic and regulatory mechanisms, as well as physiological roles, requires an understanding of its biochemical and kinetic properties. This report presents an analysis of these properties for DGKA. The enzyme catalyzes the phosphorylation of DiC8, and another medium-chain DAG, DiC6 (1,2-dihexanoyl-*sn*-glycerol), in a Michaelis−Menten manner. Interestingly, the kinetics of DGKA using physiologically relevant long-chain DAGs was dependent on substrate surface concentration and the detergent that was used. DGKA displayed Michaelis−Menten kinetics with respect to bulk substrate concentration (1,2-dioleoyl-*sn*-glycerol) in octyl glucoside mixed micelles when the surface substrate concentration was at or below 3.5 mol %. At higher surface concentrations, however, there was a sigmoidal relationship between the initial velocity and bulk substrate concentration. In contrast, DGKA displayed sigmoidal kinetics with respect to bulk substrate concentrations at all surface concentrations in Triton X-100 mixed micelles. Finally, we show the catalytic activity of DGKA was significantly enhanced by phosphatidylserine (PS) and phosphatidic acid (PA).

Diacylglycerol kinase (DGK)[1] catalyzes the transfer of the γ-phosphate of ATP to the hydroxyl group of diacylglycerol to generate phosphatidic acid (PA). This conversion of DAG to PA is important for phosphoinositide (PI) resynthesis within the PI cycle (*1*). Additionally, however, these kinases have the task of balancing the intracellular levels of two lipid molecules, diacylglycerol (DAG) and phosphatidic acid (PA), both well-documented second messengers. The DGK substrate, DAG, is an allosteric activator of classic and novel calcium-insensitive protein kinase Cs (PKCs α, β, γ, δ, ϵ, η, and θ), and DGK has been implicated as an attenuator of PKC activity, thereby inactivating downstream signaling pathways (*2*). In addition to PKC, DAG is also a key player in other cellular processes, including activation of guanyl nucleotide-releasing protein, Ras-GRP (*3*). The product of DGK, PA, is an activator of some signaling enzymes such as Raf-1 kinase, PKC ζ, and the protein tyrosine phosphatase, PTP1C, and has been implicated in the stimulation of DNA synthesis (*4*−*6*).

DGK homologues have been identified in various organisms, including mammals, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Dictyostelium discoideum*. There are nine known mammalian isoforms of DGKs, which are subdivided into five different groups based on their domain organization. The various classes of DGKs contain several domains, including a highly conserved catalytic domain, cysteine-rich repeats (C1), PH domains, EF-hand motifs, proline-rich domains, and ankyrin repeats. Many of these domains are shared between different isoforms.

A recent study identified a single gene in *D. discoideum* that appears to encode a protein that is structurally similar to the θ isoform of mammalian DGK (DGK-θ) and was designated DGKA (*7*, *8*). This study was designed to assess the DGK activity of DGKA. Insights into its regulation and physiological role are greatly facilitated by knowledge of the kinetic parameters and how these parameters are affected by various modulators. We report here some of the biochemical characteristics of DGKA. To fully evaluate the essential kinetic parameters, we performed kinetic experiments using both medium-chain and long-chain DAGs. We found that DGKA catalyzes the phosphorylation of both medium- and long-chain DAGs with pH optima of 7.4 and 7.0, respectively. Like DGK-θ and most other DGKs, DGKA does not exhibit a significant substrate preference in the presence of PS. Importantly, both PS and PA increase DGKA activity. In contrast, neither PIP_2 nor calcium affected the

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[1] Abbreviations: PA, phosphatidic acid; DAG, diacylglycerol; DGK, diacylglycerol kinase; PS, phosphatidylserine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, phosphoinositide; PKC, protein kinase C; PLD, phospholipase D; 18:1/18:1 DAG, 1,2-dioleoyl-*sn*-glycerol; 18:0/20:4 DAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; 18:0/18:2 DAG, 1-stearoyl-2-linoleoyl-*sn*-glycerol; 18:2/18:2 DAG, 1,2-dilinoleoyl-*sn*-glycerol; 18:3/18:3 DAG, 1,2-dilinolenoyl-*sn*-glycerol; DiC8, 1,2-dioctanoyl-*sn*-glycerol; DiC6, 1,2-dihexanoyl-*sn*-glycerol; K_m^a, apparent K_m; $K_{0.5}^a$, apparent $K_{0.5}$; $K_{m(Mg.ATP)}{}^a$, apparent K_m for $MgCl_2$; V_{max}^a, apparent V_{max}; k_{cat}^a, apparent k_{cat}; V_o, initial velocity.

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activity. The potential physiological significance of these findings is discussed.

EXPERIMENTAL PROCEDURES

Materials. *D. discoideum* medium (HL5) components and silica gel 60 TLC plates were purchased from EM Science. Cytoscint scintillation counting fluid was purchased from ICN (Costa Mesa, CA). [γ-^{32}P]ATP was purchased from MP Biomedical (Irvine, CA). All of the lipids except DiC6 were purchased from Avanti Polar Lipids (Alabaster, AL). DiC6, anti-FLAG M2 affinity beads, anti-FLAG M2 monoclonal antibody, and DEAE-Sephadex A-50 were purchased from Sigma. ECH Sepharose 4B was from Pharmacia (Uppsala, Sweden). Complete Cocktail Protease Inhibitor Mix was purchased from Roche. Nucleopore membranes and components were obtained from Whatman. X-ray film was purchased from Kodak.

Maintenance of D. discoideum. *D. discoideum* stocks were kindly obtained from D. Robinson. The FLAG-DGKA plasmid was transformed into a stock of *D. discoideum* by electroporation, as described by Robinson et al. (*9*).

D. discoideum cells were grown in liter quantities in HL5 medium at 23 °C, with constant agitation at 225 rpm in an Environ Shaker by Lab-Line. To maintain stock cultures, cell density was not permitted to exceed 8×10^6 cells/mL and cultures were diluted to 1×10^6 cells/mL as required (every 3−4 days). For purification of FLAG-tagged DGKA, cells expressing the enzyme were grown to a density of 8×10^6 cells/mL prior to harvesting.

Preparation of Polyunsaturated DAG Substrates. 1,2-Dilinoleoyl-*sn*-glycerol (18:2/18:2 DAG) and 1,2-dilinolenoyl-*sn*-glycerol (18:3/18:3 DAG) were generated by cleaving the PC forms of these lipids (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine and 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine) with phospholipase C from *Bacillus cereus* (PLC) as previously described (*10*). Briefly, PC was brought up in diethyl ether and Tris-HCl with 10 mM $CaCl_2$ (pH 7.4, 4:1); 2 units of PLC/mL per 2.4 μmol of PC was added to the mixture, and the mixture was briefly gassed with nitrogen, the tube capped with Parafilm, and the mixture reacted for 2−3 h at room temperature, with vigorous shaking. The organic phase was separated from the aqueous phase by centrifugation, and the DAG was recovered from the organic phase. Quantification of the amount of DAG recovered was determined as previously described using *Escherichia coli* DGK (*11*).

Enzyme Purification. FLAG-tagged DGKA was purified essentially as described by de la Roche et al. (*7*), with some modifications. Briefly, 1 L of *Dictyostelium* at a density of 8×10^6 cells/mL was centrifuged for 10 min at 1000*g*. The pellet was gently washed with 50 mM TES (pH 7.3) and 3× Roche complete protease inhibitor cocktail mix and respun again for 10 min at 1000*g*. The *Dictyostelium* cells were then resuspended in lysis buffer containing 50 mM TES, 5 mM EDTA, 200 mM NaCl, 1 mM DTT (pH 7.3), and 3× Roche protease inhibitors. Cells were lysed by passing the lysate through a 5 μm nucleopore membrane, and the soluble fraction was collected by ultracentrifugation for 1 h at 100000*g*. The soluble fraction was precleared by incubating it with 90 μL of packed Sepharose 4B for 45 min with gentle rotation on ice to eliminate proteins that nonspecifically bind to the anti-FLAG-coupled resin. The supernatant containing the expressed FLAG-DGKA was collected by centrifugation (1000*g*) for 5 min and then incubated with anti-FLAG M2 affinity agarose (per the manufacturer's protocol) for 2 h at 4 °C with gentle mixing. The beads were collected by centrifugation (1000*g*) for 5 min and washed six times with 50 mM TES (pH 7.0), 400 mM NaCl, 1 mM DTT, and 1 mM EDTA. The FLAG-DGKA was eluted from the beads by incubation with 150 μL of FLAG peptide (200 μg/μL) and collected by centrifugation (1000*g*) for 5 min. The eluate was then incubated for 1 h with gentle shaking at 4 °C with 40 μL of packed DEAE-Sephadex which removed the remaining contaminants. The beads were centrifuged at 1000*g* for 5 min, and the resulting supernatant, containing purified FLAG-DGKA, was collected and stored at −80 °C for further use. Under these conditions, DGKA activity was stable for at least 1 month (data not shown). This process yielded a protein that was greater than 95% free of other contaminating proteins (data not shown). We should note that Western blot analysis, using an anti-FLAG M2 monoclonal antibody, of the proteins in both the soluble and insoluble fractions showed that approximately 10% of the expressed DGKA is membrane-associated.

Enzyme Assays. Two DGK assays were used to test for DGK activity, depending on the substrate that was utilized. For medium-chain substrates (DiC6 or DiC8), the lipids were dried down and resuspended in 80 μL (per each reaction) of reaction buffer containing a final composition of 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM *n*-octyl β-D-glucopyranoside (OG), 1 mM DTT at pH 7.0 (for DiC6) or pH 7.4 (for DiC8), and 4 mM DAG substrate unless otherwise indicated. It should be noted that this OG concentration is well below the CMC (critical micellar concentration, 25 mM) and was added to enhance the solubility of the medium-chain DAGs. The lipid solution was vortexed and sonicated for 5 min to obtain a clear solution; 10 μL of purified FLAG-tagged DGKA and 10 μL of 1 mM [γ-^{32}P]ATP were then added to start the reaction in a 37 °C water bath for 10 min. It should be noted that all experiments using [γ-^{32}P]ATP were carried out using a protective shield, in a well-ventilated fume hood. All radioactive waste was discarded according to federal guidelines.

The long-chain substrates used in this study were 18:1/18:1 DAG, 18:0/20:4 DAG, 1-stearoyl-2-linoleoyl-*sn*-glycerol (18:0/18:2 DAG), 18:2/18:2 DAG, and 18:3/18:3 DAG, each presented to the enzyme in a micelle. Individual substrates were dried under a stream of nitrogen and brought up in a reaction buffer. For the OG micelles, the buffer contained final concentrations of 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM NaF, 1 mM DTT, 50 mM OG, and 1 mM [γ-^{32}P]ATP in a 100 μL total reaction volume. For Triton X-100 micelles, Triton X-100 was substituted for OG, while altering the 18:1/18:1 DAG concentration to maintain the desired surface concentration. To obtain different surface concentrations (mole percent) of DAG, a fixed bulk concentration of DAG was mixed with different total detergent concentrations, above the CMC of the detergent (25 mM for OG or 0.3 mM for Triton X-100). In this protocol, increases in the total amount of detergent resulted in decreases in surface concentrations of DAG. In both mixed-micellar detergent systems, the substrate (DAG) and PS surface concentrations were 6.2 and 8.1 mol %, respectively,

unless otherwise noted. The reaction was started by the addition of 10 μL of protein and 10 μL of 1 mM [γ-^{32}P]-ATP to the mixed micellar solution which was briefly vortexed and incubated in a heated water bath at 37 °C for 10 min, unless otherwise indicated.

All reactions were terminated by the addition of chloroform, methanol, and 1% perchloric acid (1:2:0.75), and the organic phase was washed twice with 1% perchloric acid. The organic phase, containing the lipids, was removed from the aqueous phase, dried under nitrogen gas, resuspended in 25 μL of chloroform and methanol (95:5), and an aliquot was spotted onto a silica gel 60 TLC plate. The lipids were separated in a solvent system composed of chloroform, acetone, methanol, acetic acid, and water (10:4:3:2:1). The plate was dried, and the produced PA was identified by autoradiography. The PA was removed from the silica gel, and the amount of [γ-^{32}P]PA was quantified by scintillation counting.

Kinetic Analysis. For DiC6 and DiC8, DAG assays were performed as described above, using various concentrations of either substrate. The initial velocity (V_o) with respect to substrate concentration was plotted, and the V_{max}^a and K_m^a parameters were determined using two methods, the Hanes plots and Sigma Plot version 8.0, by fitting the curves to a two-parameter hyperbola using the line fitting function.

For long-chain DAGs, which are highly water insoluble, assays were performed as described in Experimental Procedures. Activity was measured at various bulk substrate concentrations while a constant micellar surface composition was maintained to assess the effect of varying the bulk DAG concentration (millimolar). OG and Triton X-100 micelles were used in separate assays. The PS level was kept constant at 8.1 mol % unless otherwise indicated. Line fitting for sigmoidal curves was done using Sigma Plot by fitting to a three-parameter Hill equation:

$$V_o = \frac{V_{max}[S_o]^n}{(K_{0.5})^n + [S_o]^n}$$

where $[S_o]$ represents the bulk substrate concentration, $K_{0.5}$ represents the substrate concentration at which the initial velocity is one-half of V_{max}^a, and n is the Hill coefficient.

RESULTS

DGKA Catalyzes the Phosphorylation of Medium-Chain DAGs. In addition to the medium-chain DAG DiC8 (*7*), DGKA also catalyzed the phosphorylation of DiC6 (Figure 1a). The pH optimum was similar for both medium-chain substrates: pH 7.0 and 7.4 for DiC6 and DiC8, respectively (Figure 1b,c). The specific activity of DGKA for both substrates was also similar: 41.8 ± 0.5 nmol min^{-1} mg^{-1} at pH 7.0 for DiC6 and 51.1 ± 0.1 nmol min^{-1} mg^{-1} for DiC8 at pH 7.4 (Figure 1a). DGKA exhibited K_m^a values of approximately 148 μM for the solubilized DiC6 and 336 μM for the solubilized DiC8 (Figure 2a,b). A kinetic analysis showed the V_{max}^a values for DiC6 were 40 ± 3 and 100 ± 20 nmol min^{-1} mg^{-1} for DiC8 (Figure 2a,b), which translated into k_{cat}^a values of 0.07 and 0.2 s^{-1}, respectively. We should note that in Figure 1, the specific activity of DGKA phosphorylation of DiC8 is 51.1 nmol min^{-1} mg^{-1}, which

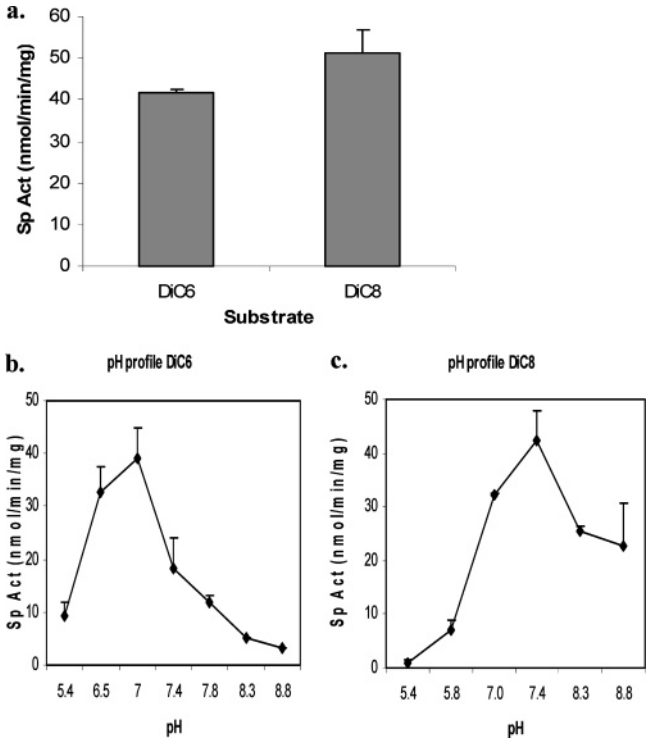

FIGURE 1: Phosphorylation of medium-chain diglycerides, DiC8 and DiC6, by DGKA, shows a physiological pH optimum. (a) DGKA activity was assessed using 4 mM DiC6 or 4 mM DiC8 as the substrate as described in Experimental Procedures. The data reported are an average of three experiments performed in duplicate. Error bars indicate the standard error. (b and c) DGKA activity using either 4 mM DiC6 or 4 mM DiC8 was measured essentially as described in Experimental Procedures, except different buffers were used to obtain a pH range from 5.4 to 8.8. The buffers used were 50 mM MES (pH 5.3 and 5.5), 50 mM Hepes (pH 6.5−7.4), and 50 mM Tris (pH 7.9−8.8). The data reported are an average of two experiments performed in duplicate, with error bars indicating the standard error.

is lower than the reported V_{max}^a for DiC8 (100 ± 20 nmol min^{-1} mg^{-1}). This apparent discrepancy exists because the experiment presented in Figure 1 was performed at 4 mM DiC8, and we have found the apparent activity decreases slightly above 1.5 mM because of difficulty in solubilizing this substrate. This experiment in Figure 1 was performed only to determine whether the enzyme phosphorylated these substrates, while the study presented in Figure 2 is a full kinetic analysis.

As noted in Experimental Procedures, 10 mM OG was included for activity measurements of either DiC6 or DiC8. This concentration is well below the CMC of OG (25 mM) and was added to keep the medium-chain DAGs dispersed. The presence of 10 mM OG did not inhibit DGKA activity (data not shown), in contrast to that seen with cytosolic DGK-α (*12*).

DGKA Catalyzes the Phosphorylation of Long-Chain DAGs. The observation that DGKA metabolizes medium-chain DAGs is consistent with its previously reported structural similarity to a mammalian DGK (DGK-θ) (*7*). The medium-chain substrates, however, are not physiological. To further characterize the DAG kinase activity of DGKA, we examined the catalytic activity using a variety of more physiologically relevant substrates with various degrees of saturation: 18:0/20:4 DAG, 18:1/18:1 DAG, 18:2/18:2 DAG, 18:3/18:3 DAG, and 18:0/18:2 DAG. As described in

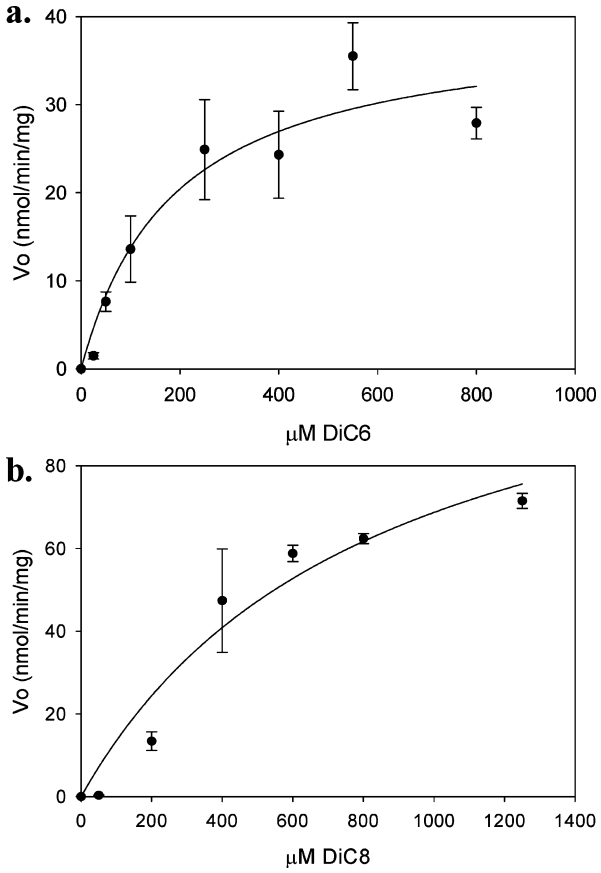

FIGURE 2: Kinetics of DGKA-mediated phosphorylation of DiC6 and DiC8. (a) The effect of DiC6 substrate concentration on initial velocity (V_o) was assessed. The concentration of DiC6 was varied as indicated in the presence of 10 mM OG as described in Experimental Procedures. The data reported are an average of two experiments performed in duplicate. Error bars indicate the standard error. (b) The effect of DiC8 substrate concentration on initial velocity was assessed as described above.

Experimental Procedures, the substrates were presented to the enzyme in an OG mixed micelle.

The data in Figure 3 clearly indicate that long-chain DAGs are also substrates for this enzyme. The pH optimum for all long chain substrates was pH 7.0 (Figure 3). Interestingly, while DGKA catalyzed the phosphorylation of all long-chain DAGs that were examined, a higher specific activity was observed for DAGs containing two polyunsaturated fatty acids, 18:2/18:2 DAG and 18:3/18:3 DAG. DGKA catalyzed the phosphorylation of 18:2/18:2 DAG with an activity 2.5-fold higher than that for a DAG containing two monounsaturated fatty acids (18:1/18:1 DAG), 3-fold higher than that for a DAG with one saturated fatty acid and one diunsaturated fatty acid (18:0/18:2 DAG), and 10.6 times higher than that for a DAG containing one saturated fatty acid and one tetraunsaturated fatty acid (18:0/20:4 DAG). The lowest specific activity was observed with 18:0/20:4 DAG as the substrate (Figure 3).

Effect of Anionic Lipids and Calcium on DGKA Activity. Anionic lipids are known to affect the activity of a variety of enzymes (*13*). Because PS (*14*, *15*) and other anionic lipids (*16*) are known modulators of DGK, we examined the effect of PS, PA, and PIP_2 on DGKA activity. As shown in Figure 4, the presence of PS (8.1 mol %) in the DAG/OG micelle enhanced the activity of DGKA as compared to that seen with only DAG in the micelle. The increase in DGKA

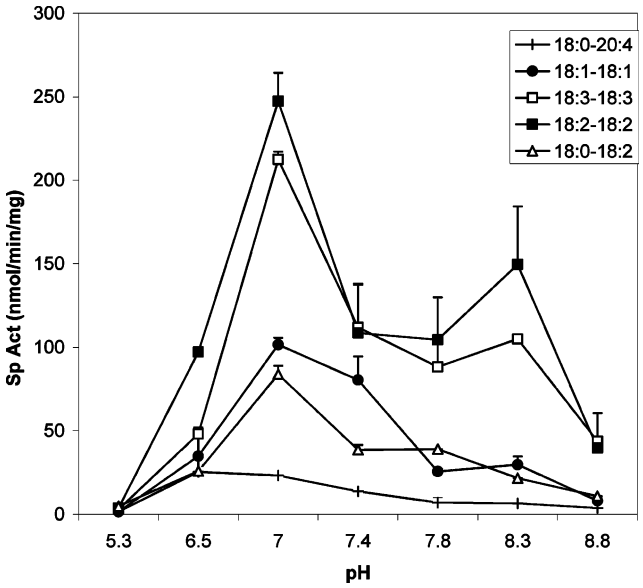

FIGURE 3: pH dependence of DGKA activity using long-chain DAG substrates. DGKA activity was quantified in OG mixed micelles containing 8.1 mol % PS and each substrate at 6.2 mol %. The substrates used were 1-stearoyl-2-arachidonoyl-*sn*-glycerol (18:0/20:4 DAG), 1,2-dioleoyl-*sn*-glycerol (18:1/18:1 DAG), 1,2-dilinolenoyl-*sn*-glycerol (18:3/18:3 DAG), 1,2-dilinoleoyl-*sn*-glycerol (18:2/18:2 DAG), and 1-stearoyl-2-linoleoyl-*sn*-glycerol (18:0/18:2 DAG). The assay pH was varied as described in the legend of Figure 1. The data reported are an average of three experiments performed in duplicate. Error bars indicate the standard error.

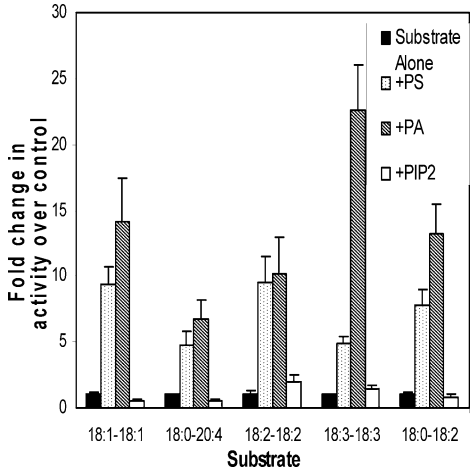

FIGURE 4: Effect of anionic lipids on DGKA activity. DGKA assays were performed in OG mixed micelles containing 8.1 mol % PS and each indicated substrate at 6.2 mol %. For each substrate, the control (black bar) indicates the DGKA assay in OG containing 6.2 mol % DAG substrate only. Each additional bar represents the addition of either 8.3 mol % PS (stippled bar) or PA (striped bar) and 0.1 mol % PIP_2 (gray bar). The data are presented as the fold difference in activity between the control and the activity when either PS, PA, or PIP_2 was included. The data reported are an average of three experiments performed in duplicate. Error bars indicate the standard error.

activity was dependent on the substrate and ranged between 6- and 10-fold. For example, activity in the presence of PS increased ~10-fold for 18:2/18:2 DAG, whereas when 18:0/18:2 DAG, 18:1/18:1 DAG, and 18:0/20:4 DAG are used, the activity was increased between 5- and 11-fold. The presence of PA (8.1 mol %) also resulted in an increase in DGKA activity for all substrates. Interestingly, however, PA appears to preferentially enhance the activity of DGKA

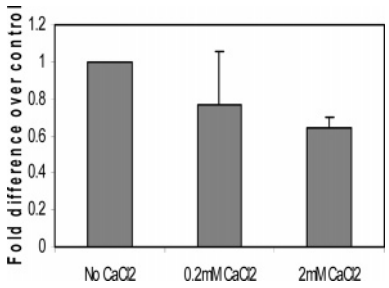

FIGURE 5: Effect of calcium on DGKA activity. DGKA activity was quantified in OG mixed micelles using 18:1/18:1 DAG (6.2 mol %) in the presence or absence of added calcium ($CaCl_2$). Calcium was added to a final concentration of 0.2 or 2 mM. The data are presented as the activity in the presence of calcium relative to the activity in the absence of added calcium. The data reported are an average of one experiment performed in triplicate. Error bars indicate the standard deviation.

toward 18:3/18:3 DAG (Figure 4). Conversely, inclusion of phosphatidylinositol 4,5-bisphosphate (PIP_2) (0.1 mol %) did not affect activity relative to the DAG substrate alone (Figure 4).

A signature of type I DGKs (DGK-α, DGK-*β*, and DGK-*γ*) is that they are calcium-sensitive (*17*). DGK-*θ*, however, is not calcium-sensitive. To further explore the relationship between DGKA and DGK-*θ*, we examined the effects of calcium on DGKA activity. DGKA activity was quantified in our standard OG assay (see Experimental Procedures) containing PS (8.1 mol %) and DAG (6.2 mol %) in the presence or absence of 0.2 or 2 mM calcium ($CaCl_2$). As shown in Figure 5, calcium had little effect on DGKA under these conditions, suggesting DGKA is not a calcium-sensitive enzyme. This is consistent with its structural similarity to DGK-*θ*.

Kinetic Analysis of DGKA: ATP and Long-Chain DAGs. During the conversion of DAG to PA, DGKA utilizes two substrates, a water-soluble substrate, ATP, and a water-insoluble substrate, DAG. Each of these substrates binds to the enzyme with different binding affinities. To determine the binding constant (K_m^a) for ATP, we varied the ATP concentration at a constant DAG concentration and examined the effect on catalytic activity. This analysis revealed a $K_{m(Mg,ATP)}^a$ of 136 ± 20.0 μM (Figure 6).

Determining the kinetic parameters for the water-insoluble substrates, (long-chain) DAGs, is more challenging because the substrate must be presented to the (soluble) enzyme in a hydrophobic environment. Kinetic examination of DAG, therefore, must involve a system where the bulk concentration (expressed in terms of molarity, millimolar) and surface concentration (expressed in terms of mole fractions, mole percent) of the lipid substrate can be varied. To accomplish this, we evaluated the kinetic parameters of DGKA using long-chain DAGs in a substrate−detergent mixed micellar system. In such systems, the bulk and surface substrate concentrations can be varied without disturbing the micellar structure within a limited dynamic range that is a function of the detergent system (approximately 15 mol % for Triton X-100 micelles) (*18*). This has been a very effective method for analyzing a variety of other enzymes that act on hydrophobic substrates (*19*).

Kinetic properties of DGKA-mediated phosphorylation of a long-chain DAG were quantified using 18:1/18:1 DAG in the presence of 8.1 mol % PS. Two detergent systems were

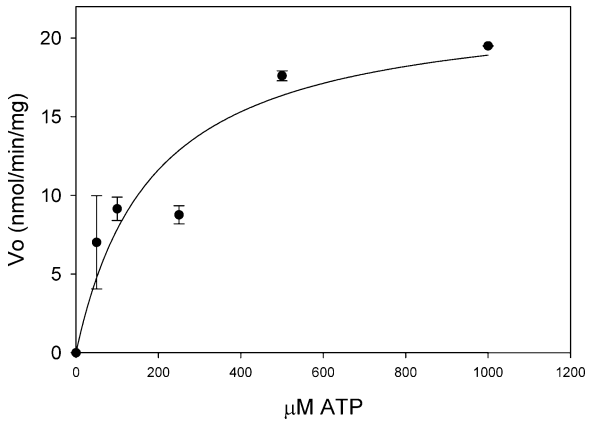

FIGURE 6: Kinetics of ATP. The $K_{m(Mg,ATP)}^a$ was determined for DGKA. DGKA initial velocities (V_o) were quantified at various ATP concentrations in OG mixed micelles containing 18:1/18:1 DAG (6.2 mol %) and PS (8.1 mol %). The data reported are an average of three experiments performed in duplicate. Error bars indicate the standard error.

examined: an OG mixed micellar system and a Triton X-100 mixed micellar system. While both systems were useful for determining kinetic parameters, there were significant differences between the two assays. First, the specific activity of the enzyme was significantly higher when assayed using Triton X-100 mixed micelles than in mixed micelles containing OG. We should note a DAG concentration from 0 to 0.6 mM was used in the OG system, as compared to a DAG range from 0 to 0.12 mM for the Triton X-100 system. The DAG concentrations in the OG system were used because DAG concentrations below 0.12 mM resulted in minimal activity which made it difficult to accurately assess the activity. Second, the substrate surface concentration had different effects on the relationship between the bulk substrate concentration and initial velocities. In mixed OG micelles, initial velocities increased in a hyperbolic manner as the bulk concentration was increased when the surface concentration was at, or below, 3.5 mol % (Figure 7a,b). These data indicate the enzyme had a V_{max}^a of 5.4−7.1 nmol min^{-1} mg^{-1} and a K_m^a of approximately 0.2−0.3 ± 0.02 mM (bulk concentration), in OG below 3.5 mol % DAG (Table 1). In contrast, when the surface concentration was increased above 3.5 mol %, sigmoidal curves were obtained (Figure 7c,d). Under these conditions, the enzyme had a V_{max}^a of 50−120 nmol min^{-1} mg^{-1}, a $K_{0.5}^a$ of 0.3 mM, and a Hill coefficient of 1.6−1.9 (Table 1).

Interestingly, when the kinetics of DGKA-mediated phosphorylation of 18:1/18:1 DAG was examined in the Triton X-100 micellar system, sigmoidal curves were observed at both low and high substrate surface concentrations (Figure 8a,b). These data were also analyzed according to the Hill equation and indicated a V_{max}^a of 200−300 nmol min^{-1} mg^{-1}, a $K_{0.5(DAG)}^a$ of 0.06−0.07, and a Hill coefficient of 3.5−4.4 (Table 1). The data indicate that DGKA is more efficient in the Triton X-100 system. The apparent k_{cat}^a/K_m^a (or $k_{cat}^a/K_{0.5}^a$) values in the OG system increased from 0.04−0.05 at low mole fractions to 0.3−0.6 at high mole fractions (Table 1). In contrast, in the Triton X-100 system, the $k_{cat}^a/K_{0.5}^a$ values are similar at both low (1 mol %) and high (6 mol %) surface substrate concentrations, and are 10-fold greater than the k_{cat}^a/K_m^a values in the OG system (Table 1).

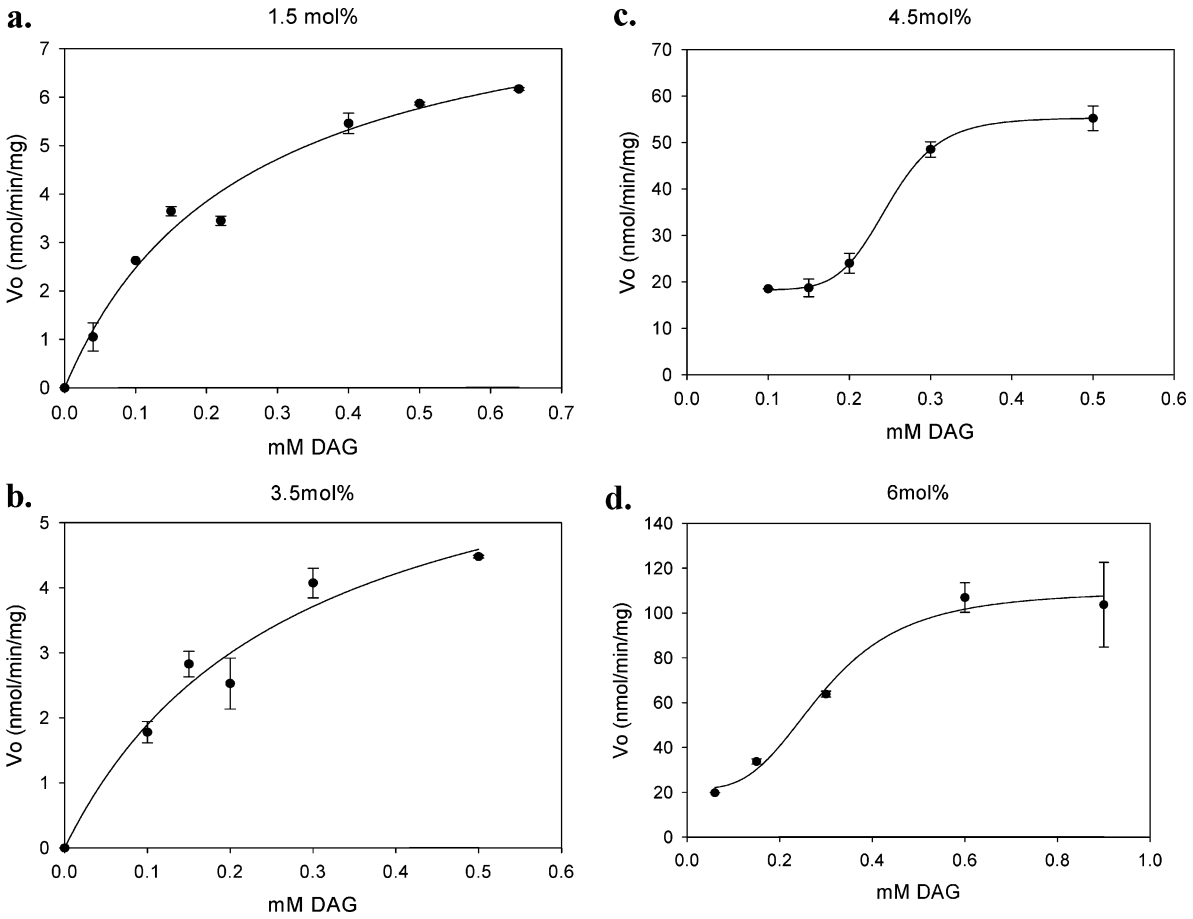

FIGURE 7: Kinetic properties of DGKA using long-chain 18:1/18:1 DAG substrate in OG mixed micelles. DGKA initial velocities (V_o) were quantified at the indicated bulk substrate (DAG) concentrations in OG mixed micelles containing 8.1 mol % PS. This analysis was performed at DAG surface concentrations of (a) 1.5, (b) 3.5, (c) 4.5, and (d) 6 mol %. The data reported are an average of two experiments performed in triplicate. Error bars indicate the standard deviation.

Table 1: Kinetic Parameters of DGKA[a]

	$K_m{}^a$ (mM)	$K_{0.5}{}^a$ (mM)	$k_{cat}{}^a$ (s^{-1})	$k_{cat}{}^a/K_m{}^a$	Hill coefficient
OG/DAG micelles					
1.5 mol %	0.22 ± 0.01	NA[b]	0.01 ± 0.003	0.05 ± 0.01	NA[b]
3.5 mol %	0.32 ± 0.05	NA[b]	0.01 ± 0.003	0.04 ± 0.0006	NA[b]
4.5 mol %	NA[b]	0.26 ± 0.01	0.09 ± 0.03	0.33 ± 0.09	1.6
6 mol %	NA[b]	0.35 ± 0.08	0.21 ± 0.03	0.61 ± 0.05	1.9
Triton X-100/DAG micelles					
1 mol %	NA[b]	0.074 ± 0.004	0.45 ± 0.09	6.1 ± 1.0	3.5
6 mol %	NA[b]	0.057 ± 0.003	0.35 ± 0.055	6.3 ± 1.3	4.4

[a] The values representing the $K_m{}^a$, $K_{0.5}{}^a$, $k_{cat}{}^a$, and $k_{cat}{}^a/K_m{}^a$ for 18:1/18:1 DAG in OG or Triton X-100 mixed micelles containing 8.1 mol % PS are presented. Error bars indicate the standard deviation. [b] Not applicable.

DISCUSSION

This study reports the first enzymatic characterization of DGKA, an enzyme in *D. discoideum* that is homologous in sequence to mammalian DGK-θ (*7*). This homology is characterized by the presence of three cysteine-rich domains, a proline-rich motif, and a catalytic domain that is 46% identical (56% similar) to DGK-θ (*7*, *20*). The cysteine-rich domains have been implicated in lipid binding (*21*) and protein−protein interactions (*20*), and the proline-rich domains contain a conserved sequence found in SH3 domain-binding proteins.

The data reported here clearly demonstrate that DGKA can catalyze the ATP-dependent phosphorylation of both medium-chain (DiC6 and DiC8) and long-chain DAGs. The pH optimum for all substrates was similar at pH 7.0. The reason for the slightly higher pH optimum for DiC8 (pH 7.4) is unclear. It is also interesting to note that there appeared to be a second pH optimum around pH 8.4 when the long-chain, dipolyunsaturated DAGs were used as substrates (Figure 3). This is similar to the two pH optima observed for DGKs found in erythrocyte membranes (*22*). The $K_{m(Mg,ATP)}{}^a$ (136 ± 20.0 μM) is well within the range observed for other kinases, including DGKs (*23*−*26*). Additionally, the activity was not increased in the presence of calcium.

PS is known to affect the activity of mammalian DGKs (*14*, *15*). PS increases the activity of most DGK isoforms (DGK-α, DGK-β, DGK-γ, DGK-ζ, DGK-ι, and DGK-θ) (*16*, *27*, *28*), while it inhibits the activity of DGK-ϵ and DGK-δ (*16*, *29*). The data in this study show that PS increases DGKA activity 5−11-fold when presented at a

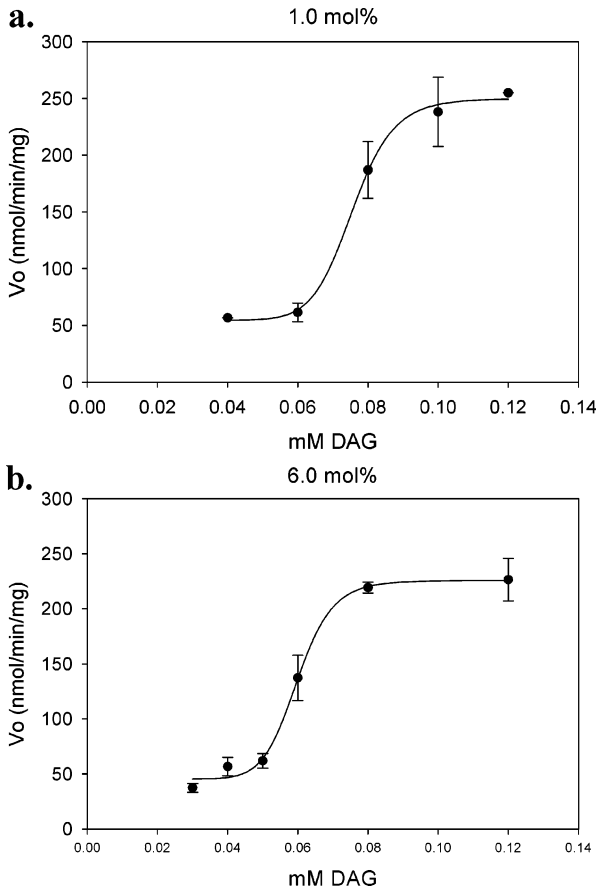

FIGURE 8: Kinetic properties of DGKA using long-chain 18:1/18:1 DAG substrate in Triton X-100 mixed micelles. DGKA initial velocities (V_o) were quantified at the indicated bulk substrate (18:1/18:1 DAG) concentrations in Triton X-100 mixed micelles containing 8.1 mol % PS. This analysis was performed at 18:1/18:1 DAG surface concentrations of (a) 1 and (b) 6 mol %. The data reported are an average of two experiments performed in triplicate. Error bars indicate the standard deviation.

surface concentration of 8.3 mol % in an OG mixed micelle (Figure 4). In our studies, PA also increased the specific activity of DGKA (Figure 4). The exact mechanism responsible for the increased activity in the presence of these anionic lipids is not clear. It is possible that the presence of these lipids results in an increase in the $V_{max}{}^a$ or a decrease in the $K_m{}^a$ of the enzyme. Additionally, these anionic lipids may enhance the binding of enzyme to the micelle (*14*, *27*). It is also interesting to note that the activity toward 18:3/18:3 DAG was enhanced to a significantly greater extent by PA than by PS (Figure 4). The precise mechanism by which these anionic lipids affect the catalytic activity toward these substrates and their physiological roles requires further study.

In addition to PS and PA, PIP_2 has been shown to activate DGK-ζ and inhibit DGK-ϵ (*16*). In contrast to PS and PA, however, PIP_2 did not have any effect on the enzymatic activity of DGKA (Figure 4). We should note that 0.1 mol % PIP_2 was used in the assay due to a technical difficulty in obtaining a clear micellar solution at PIP_2 surface concentrations at or greater than 0.5 mol % PIP_2. Importantly, this concentration (0.1 mol %) has been shown to affect the activity of DGK-ϵ (*23*) and phospholipase D from *Arabidopsis* (*30*), and is within the physiological range of this lipid. Taken together, these data suggest that DGKA may not be responsive to changes in endogenous levels of PIP_2 and therefore may not participate in the PI cycle in this organism.

One of the intriguing findings in this study was the increased specific activity with DAGs which contain at least two polyunsaturated fatty acid constituents (18:2/18:2 DAG and 18:3/18:3 DAG) (Figure 3). To date, DGK-ϵ is the only mammalian isoform that shows a preference for *sn*-2-arachidonoyl DAGs in vivo and in vitro (*15*, *25*, *31*, *32*). It has been suggested that such preferences may be physiologically important as it has been shown that the ability of DAG and phosphatidic acid to modulate certain activities likely depends on their specific molecular species (*33*−*38*). As shown in Figure 3, it was observed that DGKA had the highest specific activity with di-polyunsaturated substrates, 18:2/18:2 DAG and 18:3/18:3 DAG, and the lowest specific activity with 18:0/20:4 DAG. While the precise reason for this difference is unclear, Walsh and Bell showed that DAG exchange is not rate-limiting when *E. coli* DGK (an integral membrane protein) was assayed in an OG mixed micelle system using long-chain DAGs (*39*). While our data might lead to the conclusion that DGKA has a preference for substrates containing two polyunsaturated fatty acids, definitive assessment of this preference requires quantification of $k_{cat}{}^a/K_m{}^a$ values for all substrates as well as the potential role of differences in exchange rates. Preliminary studies with 18:2/18:2 DAG, however, indicate that under the initial velocity conditions described in this study the $k_{cat}{}^a/K_m{}^a$ value is similar to that observed with 18:1/18:1 DAG, suggesting a lack of substrate specificity. It is interesting to note that although the composition of endogenous DAGs in *D. discoideum* is not known, 18:0, 18:1, and 18:2 comprise approximately 62% of the total fatty acids (*40*) and 79% of the fatty acids in the plasma membranes (*41*). They do not, however, contain any 18:3 fatty acids (*40*, *42*). As the first position fatty acid in phospholipids and DAGs is usually saturated or monounsaturated, these data suggest that in addition to the lack of 18:3/18:3 DAGs, *Dictyostelium* probably do not contain any 18:2/18:2 DAGs. It seems, therefore, there is no physiological advantage for the organism to have a DGK with a preference for these dipolyunsaturated substrates. Further studies are in progress to definitively address these questions.

In the analysis of the kinetic parameters of DGKA, it should be noted that because we were unable to determine the disassociation constant of the enzyme from the micelle, its effect on $K_m{}^a$ and $V_{max}{}^a$ cannot be ruled out. Therefore, we report all values as apparent. In this study, it was shown that enzyme activity is dependent on the bulk substrate concentration. In addition, DGKA is also dependent on surface concentration in the OG mixed micelles. Interestingly, determination of DGKA kinetic parameters using 18:1/18:1 DAG in the two mixed micellar systems demonstrated that the absolute kinetic values were dependent on the detergent used for the analysis. In the OG mixed micelles, there was a hyperbolic relationship between the initial velocities and total (bulk) substrate concentration when the surface concentration was at or below 3.5 mol %. When the surface concentration was above this value, the relationship between the initial velocities and bulk substrate concentration was sigmoidal. A similar relationship was seen in work done on DGKI, in which the relationship between DGK activity and lipid concentration was sigmoidal (*27*). In the Triton

X-100 mixed micelles, the relationship between the initial velocities and bulk substrate concentration was sigmoidal at all surface concentrations. The sigmoidal nature of these curves suggests a positive cooperativity as the substrate concentration is increased as indicated by the positive Hill coefficients. Consistent with these observations, DGKA appears to be more efficient in the Triton X-100 mixed micelles as reflected in the increased $k_{cat}^{a}/K_{0.05}^{a}$ values. The $k_{cat}^{a}/K_{0.05}^{a}$ values were at least 10-fold higher in the Triton X-100 system than in the OG mixed micellar system (Table 1). We should note, however, that while the highest k_{cat}^{a} value observed for DGKA ($\approx$0.4 s^{-1}) is well below that observed for the *E. coli* DGK (6−12 s^{-1}) (*43*), it is well within the range observed for other kinases such as p38 mitogen-activated protein kinase (k_{cat} = 0.05−0.3 s^{-1} depending on the substrate) (*44*) and herpes simplex virus type 1 thymidine kinase (k_{cat} = 0.35−0.46 s^{-1}) (*45*). While the reason for the relatively low turnover rate is unclear, it may in part be due to the fact that the *E. coli* enzyme is an integral protein while DGKA is a soluble enzyme that must associate with the micelles prior to binding substrate. The rate of enzyme binding to the micelles is currently not known. That is, we did not know whether DGKA behaves as a "hopper" or "scooter" (*46*). Hoppers are enzymes that have a relatively low residence time on the vesicle, while scooters have a relatively long residence time on the vesicle. A parallel study of human DGK-θ indicates that this enzyme is likely to act as a hopper in the octyl glucoside system (B. Tu-Sekine, manuscript in preparation). It is possible, therefore, that this binding step influences the measurement of the turnover rate. The rate of micelle binding may also explain some of the kinetic differences observed between the OG and Triton X-100 system.

The physiological significance of the DGKA kinetics obtained in this study emerges as one of the more interesting questions. The data suggest that the enzyme either always (as in the Triton mixed micelles) or at some substrate surface concentrations (as in the OG mixed micelles) displays positive cooperativity with respect to the bulk substrate concentration. This would have important implications for the regulation of the enzyme in vivo. There are several possible explanations for this cooperativity. First, the cooperativity may be due to the ability of cysteine-rich domains to bind DAGs (*47*). In vitro, this binding would lead to an increase in the extent of micelle binding as the bulk concentration of DAG increased. In this scheme, once a "threshold" substrate concentration is reached in vivo, the membrane-associated activity would increase in a sigmoidal fashion. Another possibility is that the DGKA-mediated production of PA within the micelle serves as an activator, thereby providing a positive feedback mechanism. Consistent with this, the data in Figure 4 indicate that including PA in the mixed micelles results in increased DGK activity. In this scheme, DGK-mediated accumulation of PA in membranes would lead to an increase in membrane-associated DGK activity. In both cases, it is not entirely clear why sigmoidal kinetics was observed at all surface concentrations in the Triton mixed micelles but only at higher substrate surface concentrations in the OG mixed micelles. This may be due to an intrinsic difference between the micelles. Alternatively, this may reflect the fact that the Triton micelles are larger than the OG mixed micelles, allowing for a greater number of substrate and product molecules per vesicle at low surface concentrations. Importantly, a complete understanding of the mechanism and role of this cooperativity in vivo will require analysis of the kinetic parameters under conditions that mimic the lipid environment, including diacylglycerol concentrations, in the subcellular compartment where DGKA is operating. Future studies will address this issue.

In summary, this study presents an analysis of the enzymatic activity of DGKA. The enzyme has a pH optimum in the neutral range and phosphorylates both medium- and long-chain substrates. DGKA is activated by PS and PA but is insensitive to added calcium and PIP_2. Both bulk and surface concentrations of DAG can affect the kinetics of DGKA activity. These data will be important for future studies aimed at identifying domains that are critical for enzymatic activity. It will be particularly interesting to identify the domains in DGKA that are essential for DAG binding and to understand the mechanism(s) responsible for the cooperativity. Additionally, while this study quantifies the enzymatic character of DGKA, its physiological activity and role(s) remain unclear. Studies are in progress to address these issues.

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