Dual Regulation of Diacylglycerol Kinase (DGK)-θ

POLYBASIC PROTEINS PROMOTE ACTIVATION BY PHOSPHOLIPIDS AND INCREASE SUBSTRATE AFFINITY*

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Background: Diacylglycerol kinases are important regulators of signaling diacylglycerol, but regulation of enzymatic activity is unclear.

Results: DGK-θ activity, but not vesicle binding, is dependent on a polybasic protein for substrate binding and an acidic phospholipid for high turnover rates.

Conclusion: DGK-θ vesicle association and catalytic activity are independently regulated.

Significance: Polybasic proteins and acidic phospholipids stimulate DGK-θ substrate binding and catalytic activity.

Diacylglycerol kinases (DGKs) modulate signaling cascades by converting diacylglycerol (DAG) to phosphatidic acid (PtdOH). DAG-dependent cellular processes are numerous and diverse, coupling to both GPCR and tyrosine receptor kinase pathways (1), and many DAG signaling cascades have been shown to be regulated by DGKs, including T-cell activation and anergy (2), synaptic vesicle fusion (3, 4), trafficking (5, 6), and gene expression (7, 8). Importantly, individual DGK isoforms have been shown to regulate specific DAG effectors (for current reviews, see Refs. 9 and 10), and DGK knock-out studies have implicated specific DGKs in hypertension (11), tumor formation (12), and epileptic seizures (13, 14) in animal models. The DGK product, PtdOH, is also a lipid second messenger, and studies during the past decade have confirmed the role of DGK-derived PtdOH in the regulation of mTOR (15), phosphatidylinositol 4-phosphate 5-kinase (16), and Rab11 effector Rab-coupling protein (17). Unfortunately, structures of the mammalian DGKs have not been determined, and little is known about the regulation of DGK activity. Based on the important role of DGKs in DAG-dependent signaling, we devised a strategy for the purification and kinetic characterization of mammalian DGK enzymes and applied it to the DGK-θ isoform.

Our current knowledge of the factors that regulate DGK-θ activity is limited. Currently, three components of its regulation have been proposed: inhibition by GTP-RhoA (18), translocation to cellular membranes (19, 20), and interaction with acidic phospholipids, in particular PtdSer and PtdOH (21, and reviewed in Ref. 22). However, these studies were conducted in intact cells or cellular lysates, making it difficult to fully evaluate enzyme regulation due to the complexity of the systems.

There are several well characterized examples of enzyme activation by polybasic proteins such as stimulation of protein phosphatase 2a by Tau (23) and activation of casein kinase 2 by the small conductance calcium-activated potassium channel (SK) (24, 25). In neurons, polybasic regions (PBRs) act as a recognition site for the endocytic adapter protein AP2 (26) and for the VGCC calcium channel (27). There is also indirect evidence to suggest that DGK-θ associates with polybasic proteins in vivo, including the localization of DGK-θ in nuclear speckle domains (regions highly enriched in polybasic splicing factors) (28) and the association of DGK-θ with the PBR-containing GTPase RhoA (29).

In this study, we show that the activity of purified DGK-θ increases in the combined presence of polybasic proteins and acidic phospholipids and that stimulation by acidic lipids is dependent on the presence of a polybasic protein. Furthermore, we observed that enzyme translocation to vesicles was not dependent on the lipid composition of the vesicle or the pres-
enone of a polybasic activator, indicating that membrane translocation is not dependent on cofactors. Significantly, these data indicate that DGK-θ membrane association and enzymatic activity are independently regulated.

EXPERIMENTAL PROCEDURES

Materials—Tubulin, bovine brain Tau, and bovine brain microtubule-associated fraction (MAPF) were purchased from Cytoskeleton (Denver, CO); histone H1 was from Calbiochem (Santa Cruz, CA); and BSA (RIA grade, fraction V), α-casein, and β-casein were purchased from Sigma. All lipids were purchased from Avanti. All other chemicals were of reagent grade.

Cells and Cell Culture—HEK293T cells (ATCC) or HEK293FT cells (Invitrogen) were grown and maintained as recommended by the supplier, with the exception that 5% FBS was used for culturing cells to slow the growth rate.

Transient Transfections—HEK cells were transfected at 60–80% confluence with DNA and polyethyleneimine at a ratio of 1:4 DNA:polyethyleneimine (w/w) in OptiMEM. DNA:polyethyleneimine solutions were incubated for 15–30 min at room temperature prior to addition to cells in high glucose DMEM/5% FBS. Cells were incubated for 2–3 days prior to harvesting for protein purification.

Constructs—Human DGK-θ cDNAs were kindly provided by Dr. W. J. van Blitterswijk (Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands). To create the 5’ affinity-tagged construct, the DGK-θ gene was transferred into pCDNA3.1 at the EcoRI and NotI sites. The Fc portion of the human IgG1 heavy chain (GI: 381145023) was modified by PCR to include the tobacco etch mosaic virus (TEV) protease recognition sequence for removal of the affinity tag following purification. The TEV-Fc sequence was inserted downstream of the enzyme sequence at the Xho/I site. The Fc portion of the human IgG1 heavy chain (GI: 381145023) was modified by PCR to include the tobacco etch mosaic virus (TEV) protease recognition sequence for removal of the affinity tag following purification. The TEV-Fc sequence was inserted downstream of the enzyme sequence at the Xho/I site. The Fc portion of the human IgG1 heavy chain (GI: 381145023) was modified by PCR to include the tobacco etch mosaic virus (TEV) protease recognition sequence for removal of the affinity tag following purification. The TEV-Fc sequence was inserted downstream of the enzyme sequence at the Xho/I site.

Preparation of Cellular Fractions—Cells were washed 2 × 20 ml in ice-cold fractionation buffer (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 lysis buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, complete protease inhibitor mix (Roche Applied Science), 1 mM Na3VO4, 10 mM NaF, 0.25% Nonidet P-40). Cells were dounced 12–15 times with a type B pestle (Kontes Glass, Vineland, NJ) in a glass homogenizer. Nuclei and cell debris were removed by centrifugation at 700 × g, 4 °C for 15 min. Postnuclear supernatants were centrifuged at 100,000 × g for 1.5 h at 4 °C. Membrane-free cytosol was supplemented with 20% glycerol prior to storage at −80 °C. The membrane pellet was resuspended in half-volume lysis buffer and dounced in a Kontes homogenizer with a type B pestle to form a homogeneous suspension prior to supplementation with 20% glycerol and storage at −80 °C.

Enzyme Purification—Cell fractions were thawed rapidly at 37 °C with gentle mixing and then immediately transferred to ice. The sample was centrifuged at 1000 × g, 4 °C for 20 min prior to affinity purification. The clarified supernatant was transferred to a fresh tube, diluted to 1–2 mg/ml, and supplemented to 1% Triton X-100. Protein A-Sepharose or protein G Dynabeads (Invitrogen) previously washed with cold fractionation buffer were added to prepared samples as a 50% slurry and incubated for 3 h at 4 °C on a rotomixer. The beads were pelleted and washed 1 × 10 ml with ice-cold wash buffer 1 (WB1: 20 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5 mM EDTA, 1% Triton X-100), 2 × 10 ml ice-cold wash buffer 2 (WB2: WB1 containing 250 mM NaCl, 1% Triton X-100) and 1 × 10 ml TEV protease buffer (55 mM Tris HCl, pH 8.0, 0.005% dodecylmaltoside), followed by a 5–10-min incubation in TEV protease buffer containing 2 mM DTI (Invitrogen). Beads were resuspended in 0.2–0.4 ml of TEV buffer containing 30 units of AcTev protease (Invitrogen) and incubated for 1 h at 16 °C or 4 h at 4 °C, with gentle mixing. The His6-tagged AcTev protease was removed from the supernatant with nickel-nitrilotriacetic acid (30 min at 4 °C). The beads were pelleted, and the supernatant (containing the purified enzyme) was transferred to a fresh tube on ice, supplemented to 50% glycerol prior to storage and immediately stored at −80 °C. Enzyme concentration was estimated by densitometry of silver-stained gels using a Multi-Imagelight Cabinet with AlphaImager software (version 5.5) (Alpha Innotech Corp., San Leandro, CA). BSA standards of similar concentration were loaded onto the same gel for estimation purposes.

Vesicle Preparation—Lipids dissolved in chloroform were dried under nitrogen and stored under vacuum at 4 °C for 2–20 h to remove residual CHCl3. Lipid films were rehydrated in DGK assay buffer (55 mM Hepes, 100 mM NaCl) for 30 min at 40 °C. During hydration, samples were vortexed and sonicated (30 s, Branson Sonicator) every 10 min to completely disperse the lipid pellet. Vesicles were formed at 37–40 °C by extrusion through a 1.0-μm polycarbonate membrane using an Avanti mini extruder per manufacturer instructions and stored at 25 °C. The amount of total lipid varied between 2–10 mM. The typical lipid composition was POPC:POPE:POPS:DOG at mole fractions of 72:25:3:1, respectively; any changes are noted in the text.

DGK Assay (Large Unilamellar Vesicles)—Large unilamellar vesicles were prepared on the day of assay. When necessary, enzyme was diluted into 20 mM Tris, pH 8.0, 0.005% dodecylmaltoside to achieve a concentration of 0.1 ng/μl prior to use. ATP/MgCl2 was added to cold vesicle solution to create the reaction mix prior to addition of enzyme and activator. Reactions were incubated for 15 min at 37 °C without agitation. The final assay contained: 50 mM Hepes, pH 7.5, 1 mM DTT, 1.5 mM MgCl2, 1 mM [γ-32P]ATP with a specific activity of 2.5 × 105 cpm/nmol unless otherwise noted in the text. Reactions were terminated by addition of chloroform/methanol/1 M NaCl (1:2: 0.8) (v/v), and phases separated by addition of 1-ml each of CHCl3 and 1 M NaCl. The organic phase was washed with 2 ml of 1 M NaCl, dried under nitrogen gas, resuspended in CHCl3;
MeOH (95:5), and spotted onto a silica gel 60 TLC plate. PtdOH was separated from other lipids with chloroform:acetone: methanol:acetic acid:water (10:4:3:2:1) (v/v). The amount of [γ-32P]PtdOH was measured by liquid scintillation spectrophotometry in a Wallac 1410 liquid scintillation counter. DGK-θ activity was quantified as nmol PtdOH min⁻¹ μg⁻¹ of purified enzyme. We note that acidification of the 1 M NaCl solution to pH 2.3–2.5 with HCl and/or supplementation of control reactions with 0.1 μM polylysine or histone H1 prior to lipid extraction did not increase the amount of radiolabeled phosphatidic acid ([32P]PtdOH) recovered from vesicles (data not shown).

**Vesicle Pulldowns**—Vesicles were formed as noted for DGK assays, with the exception that DGK assay buffer contained 170 mM sucrose, and the lipid was supplemented with 0.5–0.7% Triton X-100 or Nonidet P-40) were maintained during the purification process. In addition, stability was improved by the addition of 0.005% dodecylmaltoside, a surfactant commonly used in the stabilization of membrane-associated proteins (30). Although we have successfully purified the enzyme from both membranes and cytosol, and both are activated in a similar manner, the data presented in this report were obtained using membrane-free cytosol per confluent 15 cm plate of HEK cells. This estimate was determined by densitometry of silver stained polyacrylamide gels by comparison with BSA standards (5, 10, and 20 ng BSA) on the same gel. Although the sensitivity of silver staining was useful for reliable detection of the purified protein, this method is semiquantitative due to staining variations inherent in the silver stain method, and we therefore report specific activity values and not kcat values. The purified enzyme was prone to aggregation in the absence of salt or detergent, and therefore, these components (>100 mM NaCl, 0.2% Triton X-100 or Nonidet P-40) were maintained during the purification process. In addition, stability was improved by the addition of 0.005% dodecylmaltoside, a surfactant commonly used in the stabilization of membrane-associated proteins (30). Although we have successfully purified the enzyme from both membranes and cytosol, and both are activated in a similar manner, the data presented in this report were obtained using membrane-purified enzyme based on the assumption that this population most closely resembles the active population in vivo.

Following purification, we noted that the specific activity of the enzyme did not increase with purification, suggesting that the enzyme remained denatured or that a necessary cofactor was removed during purification. To differentiate between these possibilities, we added lysates from untransfected neuroblastoma 2a or human embryonic kidney (HEK) cells to the purified enzyme in an effort to enrich for the activating protein. None, vehicle control; N2a, neuroblastoma 2a cell lysate; HEK, HEK cell lysate. Inset: cyto, cytosol; NNM, membrane fraction; Nuc, nuclear fraction.

**RESULTS**

**Activation by Polybasic Proteins**—Affinity purification of DGK-θ produced highly purified enzyme as assessed by SDS-PAGE (Fig. 1B), with estimated yields ranging from 50–150 ng of DGK-θ recovered from membranes and 100–300 ng from membrane-free cytosol per confluent 15 cm plate of HEK cells. This estimate was determined by densitometry of silver stained polyacrylamide gels by comparison with BSA standards (5, 10, and 20 ng BSA) on the same gel. Although the sensitivity of silver staining was useful for reliable detection of the purified protein, this method is semiquantitative due to staining variations inherent in the silver stain method, and we therefore report specific activity values and not kcat values. The purified enzyme was prone to aggregation in the absence of salt or detergent, and therefore, these components (>100 mM NaCl, 0.2% Triton X-100 or Nonidet P-40) were maintained during the purification process. In addition, stability was improved by the addition of 0.005% dodecylmaltoside, a surfactant commonly used in the stabilization of membrane-associated proteins (30). Although we have successfully purified the enzyme from both membranes and cytosol, and both are activated in a similar manner, the data presented in this report were obtained using membrane-purified enzyme based on the assumption that this population most closely resembles the active population in vivo.

Following purification, we noted that the specific activity of the enzyme did not increase with purification, suggesting that the enzyme remained denatured or that a necessary cofactor was removed during purification. To differentiate between these possibilities, we added lysates from untransfected neuroblastoma 2a or human embryonic kidney (HEK) cells to the purified enzyme immediately prior to the assay. This resulted in a dramatic increase in activity from 0.5 ± 0.05 nmol/min/μg to 50 ± 1 nmol/min/μg (Fig. 1C), indicating that purified DGK-θ requires a cofactor. To determine whether the cofactor was a protein, lysates were proteinase K-treated before they were
added to the purified enzyme. We reasoned that non-protein cofactors such as metals and small molecules would be unaffected by protease digestion, whereas protein activators would be reduced or eliminated. As shown in Fig. 1C, proteinase K pretreatment strongly attenuated the stimulatory effect. We compared the activating potential of membrane-depleted cytosol, cellular membranes, and nuclear lysates to determine whether the cofactor(s) was enriched in a subcellular fraction, but all were similarly effective (Fig. 1C, inset). Taken together, these results indicate that the cofactor is either a ubiquitous protein or that multiple proteins can activate the enzyme.

To differentiate between these possibilities, we conducted a small-scale screen for potential activators. Given that DGK0-θ is primarily a neuronal enzyme, we included a ubiquitous neuronal protein, Tau, and several cytoskeletal proteins (MAPF, tubulin, and actin). In addition, we tested proteins that are traditionally considered inert (BSA, casein, and ovalbumin) to determine whether recovery of activity was due to general protein stabilization. Interestingly, BSA and ovalbumin did not activate the enzyme, whereas protamine, MAPF, and the milk protein isoform β-casein were good activators (Fig. 2A). During sequence examination of these proteins, we noted an apparent correlation between the number of lysine and arginine residues (%KR) and the stimulatory effect. For example, β-casein (6.9% KR) was a poor activator, whereas α-casein (9.8–14.0% KR for mixed isoforms) was a good activator. We compared %KR content with the induced increase in activity and observed a strong correlation ($R^2 = 0.73$) between the increase in activity and the number of basic residues within the activator sequence (Fig. 2B), suggesting that basic residues are important for activation. To test this hypothesis, we examined the effect of low molecular weight poly-L-lysine and poly-L-arginine (0.1 mM) and found that both were good activators (Fig. 2C). We also tested free L-lysine and L-arginine (up to 0.1 mM) and found that they were unable to activate the enzyme, suggesting that multivalency is an important fac-

FIGURE 2. DGK-θ activators are polybasic. A, purified candidate activating proteins were tested for their ability to stimulate activity of purified DGK-θ using large unilamellar vesicles 5–7.5 mM large unilamellar vesicles (LUV; see “Experimental Procedures”). The concentration of Tau and MAPF required to produce 50% maximum activity ($K_a^{app}$) is shown for Tau and MAPF (inset). Tau, microtubule-associated protein Tau (bovine brain); MAPF, microtubule-associated protein fraction, which contains primarily MAP-1 and MAP-2 (bovine brain). White bars indicate a Lys + Arg protein content (% KR) of < 10%; black bars indicate a % KR ≥ 10% (n = 2–4 experiments in triplicate). B, the correlation between the overall Lys + Arg content (% KR) of the candidate proteins and their activating potential was examined by plotting the % KR of a candidate activator against the fold increase in DGK-θ activity. The $K_a^{app}$ for poly-L-lysine is indicated (inset). C, low molecular weight poly-L-lysine and poly-L-arginine (0.1 μM) activate DGK-θ, whereas free L-lysine and L-arginine (100 μM) do not (n = 2–3 experiments in triplicate; error bars, S.D.).
mined the KA activity over unstimulated conditions (Fig. 3A). We then deter-
mined the KA activity was measured in the absence (white bars) and presence (black bars) of 20 mol % PtdSer (0.4 mM). S.A., specific activity. B, DGK-θ was assayed over a range of poly-L-lysine in the absence (open circles) or presence (closed circles) of 10 mol % (0.25 mM) PtdSer to determine the effect of PtdSer on the Kₐ<sup>app</sup> for the activator. Vₐ<sub>max</sub> = 6.3 ± 0.3 (no PtdSer), and 29.4 ± 1.0 (10% PtdSer) (n = 2 experiments in triplicate). C, DGK-θ activity was determined over a range of PtdSer surface concentrations in the absence (open circles) or presence (closed circles) of histone H1 to determine the effect of a PBA on the (Kₐ<sup>app</sup>) for PtdSer (n = 4 experiments completed in triplicate). n.d., not determined; error bars, S.D.

**FIGURE 3. Stimulation by phospholipids requires a polybasic activator.** A, DGK-θ activity was measured in the absence (white bars) and presence (black bars) of 20 mol % PtdSer (0.4 mM). S.A., specific activity. B, DGK-θ was assayed over a range of poly-L-lysine in the absence (open circles) or presence (closed circles) of 10 mol % (0.25 mM) PtdSer to determine the effect of PtdSer on the Kₐ<sup>app</sup> for the activator. Vₐ<sub>max</sub> = 6.3 ± 0.3 (no PtdSer), and 29.4 ± 1.0 (10% PtdSer) (n = 2 experiments in triplicate). C, DGK-θ activity was determined over a range of PtdSer surface concentrations in the absence (open circles) or presence (closed circles) of histone H1 to determine the effect of a PBA on the (Kₐ<sup>app</sup>) for PtdSer (n = 4 experiments completed in triplicate). n.d., not determined; error bars, S.D.

**Activation by Phospholipids Requires a Polybasic Cofactor**—A number of DGKs have been shown to require an acidic phospholipid, typically PtdSer, for maximum activity in vitro (22). Consistent with these reports, we have previously shown that DGK-θ exhibits a dose-dependent response to the surface concentration of PtdSer and PtdOH (21). However, our initial tests of purified DGK-θ contained PtdSer, and very little activity was observed. Given the above results, we hypothesized that a polybasic activator (PBA) may be necessary for lipid stimulation of DGK-θ. To test this, DGK-θ activity was determined using liposomes (2 mM total lipid) containing 0% or 20% PtdSer, in the presence and absence of a PBA (histone H1). Low activity was observed except when both PtdSer and histone H1 (0.1 μM) were present; these conditions produced a 35-fold increase in activity over unstimulated conditions (Fig. 3A). We then determined the Kₐ<sup>app</sup> values for PtdSer and histone H1 or polylysine in the presence or absence of the partner activator. Although the presence of histone H1 promoted a marked change in the sensitivity of the enzyme to PtdSer (from no response to a Kₐ<sup>app</sup> of 8 mol %), PtdSer had no apparent impact on the Kₐ<sup>app</sup> of polylysine (Kₐ<sup>app</sup> (histone H1) = 2.1 ± 0.7 nM with 0% PtdSer; 1.8 ± 1.0 nM with 20% PtdSer) (Fig. 3, B and C). These data suggest that the interaction between DGK-θ and a PBA occurs in the absence of PtdSer, whereas the response to PtdSer is strongly dependent on the presence of a polybasic activator.

**DGK-θ Binds PBAs in Vitro and in Vivo**—To further examine the interaction between DGK-θ and PBAs, we conducted co-immunoprecipitations (co-IPs) in the presence and absence of lipid vesicles. For these experiments, we took advantage of the fact that the affinity tag binds directly to protein A or G and used purified, Fc-tagged DGK-θ associated with beads for these experiments. DGK-θ-Fc beads were incubated with purified histone H1, Tau, or ovalbumin, washed, and separated by SDS-PAGE prior to silver stain detection of bound and unbound proteins. We found that DGK-θ pulled down histone H1 and Tau, but not ovalbumin (Fig. 4A). To determine whether DGK-θ might interact with histone H1 in the presence of a membrane, we repeated the pulldowns using reactions containing vesicles (with and without PtdSer) using FcFlagDGK-θ. We note that the Flag tag does not alter the response of the enzyme to polylysine or histone H1 in an activity assay (data not shown). Again, the enzyme associated with histone H1 but not ovalbumin, and there was no apparent change in the interaction in the presence of PtdSer (Fig. 4B). This is consistent with our observation that PtdSer does not significantly alter the Kₐ<sup>app</sup> for the polybasic activator polylysine in an activity assay. To verify that DGK-θ associates with a PBA in vivo, we co-expressed GFP-tagged DGK-θ and Tau in HEK293 cells and found that DGK-θ and Tau co-immunoprecipitated in lysates (Fig. 4C).

**Activation by Phospholipid Correlates with Interfacial Association of PBAs, Not DGK-θ**—Based on the finding that PtdSer is necessary for maximal activity but not for the association of DGK-θ with an activator (Fig. 3, B and C), we reasoned that PtdSer may recruit the enzyme to the substrate. Therefore, we examined vesicle association using several lipid compositions and found that the enzyme bound similarly to all vesicles tested, even in the absence of PtdSer (Fig. 5A). We did observe an apparent increase in binding due to PtdSer and to dioleoylglycerol (DOG), suggesting some lipid influence over membrane association, although it is not clear whether this is due to an increase in membrane affinity or to changes in vesicle structure. To further study the effect of PtdSer on membrane association and activity, we examined the dose dependence of PtdSer on activity and vesicle binding in the presence and absence of histone H1. In addition, we conducted identical experiments using PtdOH or PI(4,5)P₂ in place of PtdSer because we have previously shown that DGK-θ is strongly activated by PtdOH but not by PI(4,5)P₂ (2 mol %) (21). We reasoned that PtdSer and PtdOH may show some common characteristic not observed with PI(4,5)P₂ that would help to clarify the lipid-dependent differences in activation. Sucrose-loaded vesicles were prepared fresh for each assay on the day of use and were spiked with NBD-PtdEth to track vesicle recovery and aid in normalizing. To ensure consistency between the activity and binding assays, the same batch of vesicles was used for each set of experiments. The most striking outcome was the correlation between the dose dependence of DGK-θ activity and the membrane association of histone H1 in the presence of PtdSer or PtdOH. As shown in Fig. 5C, the increase in vesicle-associated histone H1 correlated with the increase in acidic lipid. Although this was expected based on the polybasic nature of
histone H1, it is notable that the relative increase in bound PBA but not bound DGK-\(\theta\)/H9258 paralleled the increase in enzyme activity. This suggested that histone H1 might activate the enzyme at the interface, despite the fact that PtdSer does not alter the \(K_{\text{A}}\) values for histone H1 in an activity assay (see Fig. 3). In contrast, there was no apparent correlation between enzyme activity and PBA binding in the presence of PI(4,5)P2, which promoted significant histone H1 vesicle association in the absence of stimulated activity (Fig. 5C). This is likely due to the fact that PI(4,5)P2 is more highly charged and therefore more effective at recruiting PBAs. However, it also reinforces the notion that charged lipids do not function to recruit DGK-\(\theta\)/H9258. Together, the data suggest that combined interactions between the enzyme, the PBA, and the acidic lipid are necessary for stimulation. Stimulation of activity is likely to be a charge-dependent phenomenon because all three lipids are ultimately able to stimulate to nearly the same extent (Fig. 5B).

PBA As Increase the Affinity of DGK-\(\theta\) for Substrate—Because neither PBAs nor acidic lipids appeared to stimulate DGK-\(\theta\) by recruiting the enzyme to membranes, we examined their effects on substrate affinity by measuring the \(K_m\) values for ATP and surface DOG in the presence and absence of activators. To measure the surface \(K_m\) values for DOG, we held the bulk concentration of DOG constant (0.6 mM) and varied the surface concentration by altering the proportion of the remaining lipids. Experiments were conducted ± PtdSer (9 mol %) or PBA (poly-L-lysine; 0.1 mM), and the resulting data were analyzed using non-linear regression analysis (SigmaPlot, version 12.0).

Interestingly, we were unable to measure a surface \(K_m\) value for DOG in the absence of a PBA, in the presence or absence of PtdSer, due to an inability to saturate the reaction. However, when polylysine (or histone H1) was present, we observed the expected hyperbolic response to increasing surface substrate and measured a \(K_m(S)\) for DOG of 3.0 ± 1.8 mol % (Table 1) both in the presence and absence of PtdSer. This value corresponds well with our previously determined \(K_m(N)\) for unpurified cytosolic DGK-\(\theta\) (21). The \(K_m(\text{ATP})\) was 0.5 mM in the presence and absence of PtdSer and 0.1 mM polylysine, under saturating surface (8 mol %) and bulk (> 0.3 mol %) DOG concentrations, except when PtdSer and polylysine were present concurrently. Under these conditions, the \(K_m(\text{ATP})\) decreased to 0.2 mM (Table 1). These data reveal an apparent requirement for a PBA to promote substrate binding. In addition, the decrease in the \(K_m(\text{ATP})\) in the combined presence of a PBA and an acidic lipid further suggest that these components interact simultaneously with the enzyme.

DISCUSSION

In this report, we describe the purification of DGK-\(\theta\) and show that this enzyme is dependent on a polybasic cofactor and
Acidic phospholipids for full activity. As used in this report, the term “polybasic” refers to an abundance of positively charged residues within a protein, which is typically reflected by a high isoelectric point (pI). Some proteins are overtly polybasic in nature (e.g., histones, RNA splicing factors, and many microtubule-associated proteins), whereas other proteins contain PBRs. Several protein families contain conserved PBRs, including the Ras and Rho GTPases (29), and there are a number of highly expressed, unstructured proteins (including MARCKS and GAP43), which contain PBRs that have been shown to bind to and sequester acidic lipids such as PI(4,5)P2 (31). Based on the abundance of polybasic proteins in the cell, interactions must be tightly regulated to avoid signaling calamities. In vivo, this might be accomplished by sequestration within a cellular compartment or by charge masking through posttranslational modifications (lysine acetylation) or protein interactions (e.g., inhibitory subunits or calmodulin).

The primary activators used in this study were histone H1 and low molecular weight poly-L-lysine (a synthetic polymer of lysine residues). Although polylysine is a non-physiologic molecule, we chose to use it because it provided a means of investigating the polybasic effect in the absence of other protein interactions. However, it is clear from our data that natural proteins such as histone H1 and Tau, which contain relatively short polybasic regions, are also effective activators of DGK-θ. Although contributions to activity from PBA-induced surface effects on acidic vesicles (32, 33) cannot be excluded based on this study, the observation that PBAs reduce the interfacial $K_m(S)$DOG in the absence of a negatively charged lipid suggests that the PBA directly influ-

**TABLE 1**

PBAs are necessary and sufficient for DOG binding at the interface

The $K_m$ values for ATP and DOG were determined in the presence and absence of PtdSer (8 mol % / 0.2 mM). Error bars, S.D. **, not measurable. PS, phosphatidylserine.

|                   | No activator | Polylysine |
|-------------------|--------------|------------|
| $K_m$(ATP)        | + PS         | 0.51 ± 0.05| 0.21 ± 0.01|
| $K_m$(ATP)        | No PS        | 0.46 ± 0.16| 0.51 ± 0.06|
| $K_m(S)$DOG       | + PS         | **         | 3.0 ± 0.6 |
| $K_m(S)$DOG       | No PS        | **         | 3.4 ± 1.4 |

FIGURE 5. Stimulation of DGK-θ correlates with PBA recruitment by monovalent acidic phospholipids. A, DGK-θ was incubated with sucrose-loaded vesicles of varying lipid composition in the presence and absence of histone H1. The amount of enzyme in the supernatants and pellets was determined by densitometry of silver stained gels. Columns indicate % bound enzyme in the absence (white) or presence (gray) of histone H1. PC: phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DG, dioleoylglycerol. B, high levels of PtdSer (white), PtdOH (gray), and PI(4,5)P2 (black) stimulate DGK-θ to similar extents in the presence of histone H1 (n = 3 experiments in triplicate from different batches of enzyme). Error bars, S.D. C, DGK-θ and histone H1 were incubated with sucrose-loaded vesicles containing increasing concentrations of acidic lipid. Proteins associated with vesicles were quantitated by densitometry. Dashed lines represent DGK-θ activity; columns indicate the fold increase in DGK-θ (white) or histone H1 (gray) (n = 2 – 4 experiments for each lipid using separate batches of enzyme).
ences substrate binding and not through a clustering of surface lipid charges. In vitro association of purified DGK-θ with histone H1 and Tau, conducted in the absence of lipids, supports this hypothesis (Fig. 4).

Stimulation of DGK-θ, as with any interfacial enzyme, stems from changes in one or more of the following three general factors: 1) enhanced membrane association, 2) increased affinity for substrates, or 3) an increase in the catalytic rate. Our data show that purified DGK-θ associates with phospholipid vesicles in a low activity state in a manner that is largely independent of the tested lipid and protein cofactors, suggesting that membrane recruitment is not the primary regulator of DGK-θ activity. The in vitro data are consistent with the published observation that DGK-θ translocates to cellular membranes without observable increases activity (20), further supporting this hypothesis. It is interesting to note that the proportion of vesicle-associated enzyme reported here is consistent with the relative distribution of DGK-θ we have consistently observed in cell fractions harvested from a variety of cell lines (including HEK293, Cos7, and IIC9). If the observed in vivo pattern of membrane association is due to the general lipid affinity observed in vitro, the requirement for an activating protein cofactor would impart a necessary level of regulation in the cell.

The kinetic data are consistent with individual roles for polybasic proteins and acidic phospholipids in DGK-θ activation. During this study, we found that a variety of polybasic proteins could activate the enzyme, suggesting a charge-based mechanism. This is supported by the observation that two natural polyamines, spermine and spermidine, are effective activators at 0.5–1.0 mM (data not shown). In comparison, Tau, MAPF, and histone H1, which contain clusters of basic amino acids, stimulate when present at low mM concentrations.

The contribution of the monovalent acidic phospholipids PtdSer and PtdOH appears to be 2-fold in our system: 1) charge-based recruitment of PBAs to the interface and 2) stimulation of catalytic activity. Although it is clear that Histone H1 and polylysine increased substrate affinity in the absence of an acidic lipid, addition of PtdSer further stimulated the enzyme by increasing catalytic activity. At high surface concentrations of PtdSer (15–20%), activity increased an additional 10-fold over enzyme stimulated by histone H1 only, although the mechanism of this stimulation is unclear. Our initial hypothesis was that acidic lipids recruited PBAs to the interface and that the primary mode of activation was through increased substrate affinity. However, our experiments with P(4,5)P_2, which strongly recruited Histone H1 in the absence of enzyme activation, suggest that increasing the concentration of an activator on the vesicle is not sufficient to fully activate the enzyme, though the mechanism of activation is not yet known. It is noteworthy that PtdSer was an effective activator of DGK-θ near the physiologic concentration, whereas this was not true for PtdOH or P(4,5)P_2 (see Fig. 5). Further study will be necessary to determine the mechanism of lipid-based activation.

This work examined general properties of DGK-θ regulation using purified enzyme and resulted in the discovery that polybasic proteins and monovalent acidic phospholipids are necessary for optimum activity. These cofactors synergistically activate the enzyme by enhancing substrate binding and turnover rate, respectively.

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