Multiple Factors Influence the Binding of a Soluble, Ca^{2+}-Independent, Diacylglycerol Kinase to Unilamellar Phosphoglyceride Vesicles†

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ABSTRACT: We studied the influence of membrane lipids, $MgCl_2$, and ATP on the ability of a soluble diacylglycerol kinase to bind to 100-nm lipid vesicles. The enzyme did not bind detectably to vesicles that contained phosphatidylcholine alone or to vesicles that contained 50 mol % phosphatidylcholine + 50 mol % phosphatidylethanolamine. But it did bind to vesicles that contained anionic phosphoglycerides, and maximal binding occurred (in the presence of $MgCl_2$) when the vesicles contained anionic phosphoglycerides alone. When increasing amounts of phosphatidylcholine were included in phosphatidylserine-containing vesicles, enzyme binding to the vesicles decreased by as much as 1000-fold. However, when increasing amounts of phosphatidylethanolamine were included in phosphatidylserine-containing vesicles, little change in binding occurred until the concentration of phosphatidylserine was reduced to below 25 mol %. These results and results obtained with vesicles that contained various mixtures of anionic phosphoglycerides, phosphatidylcholine, phosphatidylethanolamine, and unesterified cholesterol provided evidence that anionic phosphoglycerides were positive effectors of binding, phosphatidylcholine was a negative effector, and phosphatidylethanolamine and unesterified cholesterol were essentially neutral diluents. Other experiments showed that diacylglycerol and some of its structural analogues also were important, positive effectors of enzyme binding and that addition of ATP to the medium increased their effects. The combined results of the study suggest that the enzyme may bind to vesicles via at least two types of binding sites: one type that requires anionic phospholipids and is enhanced by Mg^{2+} but inhibited by phosphatidylcholine, and one type that requires diacylglycerol and is enhanced by ATP.

Mammalian tissues contain at least 10 different diacylglycerol kinases (DGKs),[1] as demonstrated by protein fractionation experiments (*1*−*6*) and molecular cloning studies (*7*−*20*). The DGKs are generally thought to attenuate DG signals formed during the course of stimulus transduction, generate PA signals required for other cell functions, and/or promote the recycling of PI (reviewed in refs *21*−*23*). But it seems likely that specific DGKs function in special pathways and that this accounts for the diversity that has been observed. Several experimental findings support this possibility: Studies of the mRNAs of several of the DGKs have demonstrated that they are distributed differently among various tissues and cell types (*11*−*14*). Protein fractionation experiments have shown that a single cell can contain as many as three different DGK isoforms (*3*, *5*). Analyses of the subcellular locations of several of the DGKs have provided evidence that some are mainly cytosolic, some are mainly associated with membranes, and at least one is localized to the nucleus (*1*, *4*, *12*, *14*, *24*, *25*). Finally, analyses of the reactivity of several of the DGKs have shown that some react preferentially with DGs that contain esterified 20:4 whereas others do not (*24*, *25*); some are regulated by Ca^{2+} whereas others are not (*26*); and some are regulated by additional mechanisms (*5*−*7*, *9*, *27*−*32*).

If the special functions of specific DGKs are to be understood, the properties of each one will have to be characterized in detail. For example, because DGK reactions in intact cells are likely to occur at the interface between cell membranes and the cytosol, it will be important to identify the factors that influence the abilities of the DGKs to interact with this interface. Little information concerning these factors is currently available because most studies of the reactivity of individual DGKs have used assays containing mixed micelles of detergents and DG. These assays are simple and can provide useful information (*33*), but they are also unphysiological. It might be possible to develop more

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[1] Abbreviations: DG, diacylglycerol; DGK, diacylglycerol kinase; DGK1, diacylglycerol kinase 1; DGK2, diacylglycerol kinase 2; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; K_D^{app}, apparent dissociation constant; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; 16:0, palmitoyl; 16:0−18:1-DG, *sn*-1-palmitoyl-2-oleoyl-DG; 16:0−18:1-PC, *sn*-1-palmitoyl-2-oleoyl-PC; 16:0−18.1-PE, *sn*-1-palmitoyl-2-oleoyl-PE; 16:0−18:1-PS, *sn*-1-palmitoyl-2-oleoyl-PS; 18:0, stearoyl; 20:4, arachidonoyl; 18:0−20:4-DG, *sn*-1-stearoyl-2-arachidonoyl-DG; 18:0−20:4-PC, *sn*-1-stearoyl-2-arachidonoyl-PC; 18:0−20:4-PE, *sn*-1-stearoyl-2-arachidonoyl-PE; 18:1, oleoyl; 18:1−18:1-DG, *sn*-1,2-dioleoyl-DG.

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physiologically relevant assays by first examining the interactions of DGKs with well-characterized, unilamellar lipid vesicles and then analyzing the effects of membrane proteins on these interactions. Assays with unilamellar lipid vesicles have already been used to characterize both interfacial binding and catalytic activation of other enzymes, particularly isoforms of protein kinase C and phospholipase A_2 (*34*−*37*). But assays of this type have been used in only a limited way for studies of DGKs (*38*−*40*).

In the study described below, we used a unilamellar lipid vesicle assay system to identify lipids and medium components that influence the ability of a cytosolic, Ca^{2+}-independent DGK from Swiss 3T3 cells to bind to membranes. We used stable, 100-nm diameter vesicles because they were large enough to have a curvature similar to that of planar bilayers in cells, but small enough to have a relatively uniform size distribution. We investigated the effects of different classes and molecular species of phosphoglycerides, the effects of cholesterol and DG, and the effects of Mg^{2+} and ATP. The results showed that a complex array of both membrane-associated and cytosolic factors could influence the enzyme's ability to bind to the vesicles. We report in a companion paper that the information gained about these factors can be used to develop an effective affinity purification method for the DGK and study the relation between the enzyme's binding to vesicles and its catalytic activity (*41*).

MATERIALS AND METHODS

Materials. Most phosphoglycerides and diacylglycerol were purchased from Avanti Polar Lipids; the egg PC, brain PS, liver PI, and heart PE were mixtures of phosphoglyceride molecular species, and Avanti prepared the PG and PA from egg PC. The egg PC and the egg PC-derived PG and PA mainly consisted of molecular species that contained 16:0 and 18:1. The corresponding fatty acids for brain PS were 18:0 and 18:1, and those for liver PI and heart PE were 18:0 and 20:4. 18:0−20:4-DG was purchased from Sigma; 18:0−20:4-PE was purified from bovine heart PE (Avanti) by reverse-phase HPLC. Avidin was purchased from Vector Laboratories. Dextran sulfate−Sepharose was prepared as described (*42*).

Cell Culture. Swiss 3T3 cells were maintained in culture essentially as described (42). Before being used for experiments, the cells were plated at a density of 10^4 cells/cm^2 in 5% plasma-derived bovine serum and maintained in culture for 6 days with medium changes every 2 days.

Partial Purification of DGKs from Quiescent 3T3 Cell Cytosol. All steps were performed at 4 °C. The cells were washed twice with phosphate-buffered saline containing 1 mg/mL glucose and once with buffer A (0.25 M sucrose, 25 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.5, with the following protease inhibitors: 20 μg/mL soybean trypsin inhibitor, 0.5 μg/mL leupeptin, 1.0 μg/mL pepstatin A, 0.5 μg/mL aprotinin). The cells were scraped from culture dishes in 1 mL of buffer A per 150- mm dish and disrupted in a Dounce homogenizer with five strokes of an "A" type pestle (Kontes). Nuclei and large debris were removed by centrifugation at 750*g* for 10 min. The postnuclear supernatant was centrifuged at 100000*g* for 45 min to obtain cytosol and membrane fractions.

The cytosol fraction (50 mg of protein) was loaded directly onto an 8-mL column of dextran sulfate−Sepharose. The column was washed with buffer A; then DGKs were eluted with a 60-ml gradient of 0−1.2 M NaCl in buffer A. Two peaks of activity were detected with the use of the standard vesicle assay described below or a mixed micelle assay (*24*). The first peak, which we will refer to as DGK1, emerged at 370 mM NaCl, the second, "DGK2", at 860 mM NaCl (Figure 1A). On the basis of their differential activities in mixed micelle assays (not shown), we believe that DGK1 and DGK2 correspond to the type I and II DGKs identified previously in 3T3 cells (*5*). Fractions corresponding to DGK1 and DGK2 were pooled separately, dialyzed against MOPS−NaCl buffer (50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 0.1 mM DTPA), and frozen at −70 °C. Dialyzed DGK1, purified 7−12-fold as compared with the cytosol, was used for all of the experiments in this paper.

The membrane fraction from the 3T3 cell homogenate was solubilized with 1% (19 mM) Triton X-114 at 4 °C and separated into aqueous and detergent phases by precipitation of the Triton at 37 °C followed by centrifugation (*44*). When the aqueous phase (<36 mg of protein) was chromatographed on a column of dextran sulfate−Sepharose under conditions similar to those used for the cytosol, two peaks of DGK activity eluted at the salt concentrations found for DGK1 and DGK2 from the cytosol (Figure 1B).

Preparation of 100-nm, Unilamellar Phosphoglyceride Vesicles. Lipid mixtures of various compositions were dried under argon and resuspended in buffer B. Multilamellar vesicles were formed by vortexing and, when necessary, gentle sonication in a Branson bath sonicator until all lipid was resuspended from the tube walls. Then the vesicles were frozen and thawed 5 times and forced through a 0.1-μM polycarbonate filter 10 times with the use of a Lipex Biomembranes Inc. extruder. This procedure yielded unilamellar vesicles of approximately 100-nm diameter.

All vesicles used were stable for up to 1 week after extrusion as determined by measurements of the average vesicle diameter on a Brookhaven Instruments 90$^+$ particle sizer (<30% deviation from 100 nm), or by measurements of the optical density at 350 nm (values obtained were within 2-fold of the values for the same concentration of a known 100-nm vesicle suspension). The two methods gave similar results. The vesicles did not change detectably when added to incubation assays that contained 2 mM $MgCl_2$. However, precautions had to be taken to prevent oxidation of vesicle lipids, and addition of 0.1 mM DTPA to all buffers was sufficient.

Assays of DGK1 Binding to Vesicles. Binding of DGK1 to sample vesicles containing 1 mol % biotinylated PE was measured using one of two variations of the method of Hashimoto (45). (A) For dilute suspensions of sample vesicles, DGK1 was incubated with the vesicles for 5 min in 50 μL of MOPS−NaCl buffer at 37 °C; 5 μL of 50 mM carrier vesicles containing 99 mol % PC + 1 mol % biotinylated PE was added along with 5 μL of 7.5 mg/mL avidin to precipitate the sample vesicles quickly; and after an additional 5 min, the precipitated sample and carrier vesicles were centrifuged in a tabletop microfuge for 4 min at 16000*g*. Then the DGK1 activities in the supernatant and pellet fractions were measured. Note that DGK1 did not associate detectably with the carrier vesicles. (B) For

concentrated suspensions of sample vesicles (10−32 mM), addition of carrier vesicles was not required. Instead, avidin was added directly to the sample vesicles in concentrations sufficient to yield avidin-to-biotin ratios and final volumes comparable with those used in the assays with added carrier vesicles.

DGK1 activity in the pellets was assayed by one of two methods. Either the pellets were resuspended in 60 μL of MOPS−NaCl buffer and assayed directly, or they were extracted with Triton X-114, a phase separation was subsequently performed as described above, and aliquots of the aqueous phase were assayed. Values obtained with either method were corrected to account for the contributions of pellet lipid and trapped supernatant solution to the total pellet volume. The two contributions were determined experimentally by measuring the weight of the recovered pellet and the pellet's content of radioactive ATP, respectively, and were found to be linearly related to the concentration of total lipid. The results obtained with or without Triton X-114 treatment were essentially the same, and the recovery of total DGK1 activity was usually 40−100% as long as siliconized tubes were used for the binding assays. The association of DGK1 to vesicles could be measured over a range of 10 μM to 30 mM total lipid. Above 30 mM, the vesicles could not be resuspended, and below 10 μM (0.4 μg of lipid) the protein-to-lipid ratio was too high to ensure that the lipids were not being saturated.

Calculation of Apparent Dissociation Constants and Enzyme Binding Affinities. If DGK1 exists in an equilibrium between membrane-bound and soluble forms, there should be an apparent dissociation constant, K_D^{app}, equal to the vesicle concentration times the free enzyme concentration, divided by the concentration of bound enzyme, which is mathematically equivalent to the expression for single-site binding for a ligand. We used the GraFit program from Erithicus software (*46*) and the single-site binding equation supplied with it to calculate K_D^{app} values based on measurements of the fraction of DGK1 bound at 4−7 different concentrations of vesicles, which ranged from 0.01 to 30 mM total phosphoglyceride. Vesicle concentrations were expressed in millimolar total phospholipid for simplicity, so K_D^{app} was expressed in millimolar. In addition, enzyme binding affinity was expressed as $1/K_D^{app}$. In all cases where the binding capacity could be measured accurately, it was found to be essentially 100%; i.e., all of the DGK1 associated with the vesicles at saturation. In the case of vesicles with such a high K_D^{app} that the measured binding capacity did not reach a maximum, a potential for reaching 100% capacity was assumed.

DGK1 Activity Assays. DGK1 assays contained 0.2 mM unlabeled ATP, 250−1000 cpm/pmol [γ-^{32}P]ATP, 2−5 mM $MgCl_2$, 2−10 mM (total phosphoglyceride) standard assay vesicles, and DGK1 in 50 μL of MOPS−NaCl buffer. The standard assay vesicles consisted of 52 mol % heart PE + 8 mol % 18:0−20:4-DG + 20 mol % brain PS + 20 mol % egg PC. The reaction mixtures were incubated for 10 min at room temperature and quenched with 900 μL of chloroform−methanol−concentrated HCl (66:33:1). The lipids were washed 2 times with synthetic upper phase (chloroform−methanol−water, 3:48:47) to remove unreacted ATP. The PA products were isolated on plastic-backed silica plates that had been developed in chloroform−acetone−methanol−acetic acid−water, 6:8:2:2:1 (*47*), and counted for radioactivity. The measured activity was linear with enzyme and time, and saturation was demonstrated for all reaction components, as the accompanying paper demonstrates (*41*).

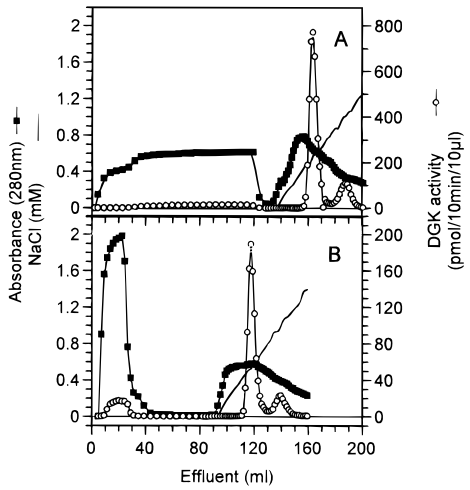

FIGURE 1: Fractionation of Swiss 3T3 cell cytosolic and membrane-derived DGKs by chromatography on dextran sulfate−Sepharose. (A) The high-speed supernatant fraction from a Swiss 3T3 cell homogenate was chromatographed on a column of dextran sulfate−Sepharose, and DGK activity was assayed by incubation for 10 min with 10 mM standard assay liposomes, 1 mM [γ-^{32}P]ATP, and 5 mM $MgCl_2$ in MOPS−NaCl buffer (Materials and Methods). (B) The membrane fraction from the Swiss 3T3 cell homogenate was extracted with Triton X-114 and subjected to phase-partitioning (Materials and Methods). Then the aqueous phase was chromatographed on dextran sulfate−Sepharose, and DGK activity was analyzed as in (A). Note that the two peaks of DGK activity respectively detected in the cytosol (A) and membrane-derived fraction (B) eluted at identical salt concentrations; that the distribution of DGK activity between the two peaks, hereafter referred to as DGK1 and DGK2, was similar in (A) and (B); and that similar results were obtained in a second experiment.

Other Methods. Protein concentrations were determined by the method of Bradford (*48*). Phospholipid concentrations were determined by phosphate assays following acid hydrolysis (*49*), and DG concentrations were determined by ester assays using cholesterol acetate as a standard (*50*).

RESULTS

Effects of Divalent Cations on the Binding of DGK1 to Anionic Phosphoglyceride-Containing Vesicles. Swiss 3T3 cells contain at least three different DGKs. One catalyzes the preferential phosphorylation of *sn*-1-acyl-2-20:4-DG (*25*) and cofractionates with membranes of the endoplasmic reticulum (*51*); the other two show no preference for this molecular species of DG and seem to be distributed between membranes and the cytosol (major and minor peaks shown in Figure 1A,B; ref *5* and *41*; and data not shown). To identify determinants that might influence the ability of the major cytosolic DGK (DGK1) to bind to cell membranes, we initially incubated the enzyme with suspensions of 100-nm unilamellar, PC-containing vesicles in a medium that contained MOPS−NaCl buffer (50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 0.1 mM DTPA), then separated the vesicles from the supernatant by centrifugation, and measured the amounts of enzyme activity recovered in each fraction (Materials and Methods). The results of these experiments revealed little enzyme binding to vesicles that

Table 1: Inactivation of DGK1 by Anionic Phosphoglycerides

	relative DGK1 activity
100 mol % brain PS	0.31 ± 0.06
100 mol % egg PG	0.23 ± 0.06
100 mol % liver PI	0.36 ± 0.01
100 mol % egg PA	0.01 ± 0.00
60 mol % brain PS + 40 mol % egg PC	1.06 ± 0.01
60 mol % egg PG + 40 mol % egg PC	1.04 ± 0.01
60 mol % liver PI + 40 mol % egg PC	1.14 ± 0.04
60 mol % egg PA + 40 mol % egg PC	0.07 ± 0.06
20 mol % brain PS + 80 mol % heart PE	1.03 ± 0.06
100 mol % brain PS, 2 mM $MgCl_2$	1.17 ± 0.03
100 mol % egg PG, 2 mM $MgCl_2$	1.09 ± 0.04
100 mol % liver PI, 2 mM $MgCl_2$	1.16 ± 0.04
100 mol % egg PA, 2mM $MgCl_2$	0.25 ± 0.00
60 mol % egg PA + 40 mol % egg PC, 2 mM $MgCl_2$	1.13 ± 0.00
100 mol % brain PS, 1 mM $CaCl_2$	1.14 ± 0.03
100 mol % brain PS, 300 mM NaCl	1.14 ± 0.00

[a] DGK1 was incubated for 5 min with the indicated vesicles (12 mM total phospholipid) and salts (in addition to the 100 mM NaCl in the MOPS−NaCl buffer). Then DGK1 was separated from the phosphoglycerides with a Triton X-114 phase separation method, and DGK1 activity was measured with the standard assay procedure. DGK1 activity was expressed relative to DGK1 activity in the absence of vesicles or salts. Similar results were observed in a second experiment.

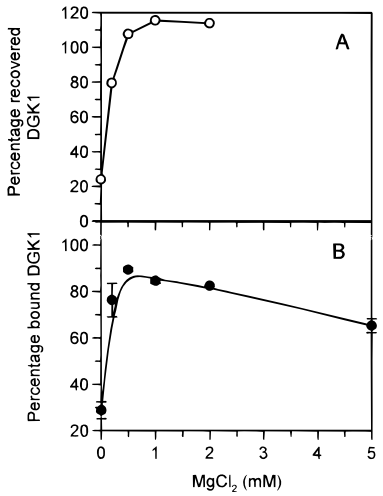

FIGURE 2: Effects of $MgCl_2$ on the PS-induced inactivation of DGK1 and the binding of DGK1 to vesicles containing egg PC and brain PS. (A) DGK1 was preincubated at 37 °C in the presence of the indicated concentrations of $MgCl_2$ and vesicles containing 100 mol % brain PS (total volume of incubation mixture, 20 μL; total concentration of vesicle phosphoglyceride, 12.5 mM). After 5 min, 80 μL of 23.25 mM 1.25% Triton X-114 was added, a phase separation was performed, and the aqueous phase was assayed for DGK1 activity. Similar results were observed in experiments with 0.5 or 2 mM $MgCl_2$. (B) DGK1 was mixed with vesicles composed of 40 mol % egg PC + 60 mol % brain PS (total concentration phosphoglyceride 3.2 mM) in the presence of the indicated concentrations of $MgCl_2$. The vesicles were then precipitated and separated from the supernatant by centrifugation. Finally, DGK1 activities in the supernatant and vesicle fractions were assayed as described under Materials and Methods. Similar results were obtained in a second experiment.

consisted of 100 mol % egg PC or to vesicles that consisted of 80 mol % egg PC + 20 mol % brain PS, but did demonstrate binding to vesicles that consisted of mixtures of egg PC + >50 mol % brain PS (data not shown). However, DGK1 lost some activity when it was incubated with vesicles that contained 20 mol % egg PC + 80 mol % brain PS (data not shown); and it lost most of its activity irreversibly within a few minutes when it was incubated with vesicles that consisted of 100 mol % brain PS (Table 1). Loss of activity was detected whether phosphoglycerides were removed before the enzyme's activity was assayed or not; liver PI and egg PG inactivated DGK1 as effectively as did brain PS; and egg PA even inactivated the enzyme at a concentration of 60 mol % (Table 1).

In subsequent experiments with DGK1 and vesicles, we discovered that no enzyme inactivation occurred when DGK1 was incubated with vesicles that contained 100 mol % PS in the presence of 0.5−2 mM $MgCl_2$ or $CaCl_2$ (Figure 2A and Table 1). Note that most of the recovered enzyme activity was bound to the vesicles under these conditions (Figure 4) and that 2 mM $MgCl_2$ had a smaller protective effect when the enzyme was incubated with vesicles that consisted of 100 mol % PA (Table 1). Mg^{2+} and Ca^{2+} probably reduced DGK1 inactivation by a mechanism that involved electrostatic shielding because 0.3 M NaCl also could prevent DGK1 inactivation by vesicles that consisted of 100 mol % PS (Table 1).

However, the enzyme showed *increased affinity* for vesicles that contained 40 mol % egg PC + 60 mol % anionic phosphoglycerides in the presence of 0.5−2 mM $MgCl_2$ or $CaCl_2$ (Figure 2B). For example, the calculated K_D^{app} (Materials and Methods) for vesicles that contained 40 mol % egg PC + 60 mol % brain PS was approximately 5-fold lower in the presence of 2 mM $MgCl_2$ than it was in the absence of $MgCl_2$ (data not shown). The molecular basis of this effect remains to be clarified, but heat-inactivation experiments with DGK1 showed that 2 mM $MgCl_2$ could partially stabilize the enzyme at 45 °C and this effect did not depend on the presence of added vesicles (Figure 3). This suggests that the divalent cations used in our experiments may have bound directly to DGK1 and influenced its structure. Divalent cations have been shown to influence the stability and structure of other enzymes (*27*, *52*, *53*).

Having demonstrated the effects of added divalent cations on enzyme binding and inactivation, we included 1−2 mM $MgCl_2$ in the incubation medium in all subsequent experiments. Though Mg^{2+} and Ca^{2+} appeared to be equally effective in vitro, the concentrations required were close to the physiological concentration of intracellular Mg^{2+}, but were far from that of intracellular Ca^{2+}. Furthermore, addition of 100 μM $CaCl_2$ to incubation media containing 0.5−2.0 mM $MgCl_2$ had no further effect (data not shown).

Effects of Anionic Phosphoglycerides on Enzyme Binding to PC-Containing Vesicles. When we incubated DGK1 with vesicles that contained various mixtures of egg PC + brain PS in the presence of $MgCl_2$, we found that the enzyme's ability to bind to the vesicles increased as a function of the relative content of PS in the vesicles. Calculated values for K_D^{app} decreased from >200 mM for vesicles that contained 100 mol % egg PC or vesicles that contained 80 mol % egg PC + 20 mol % brain PS to 0.2 mM for vesicles that contained 100 mol % brain PS. Furthermore, for any 2-fold increase in the mol % of brain PS, there was a much greater change in K_D^{app} (Figure 4). Therefore, the effects of brain PS were highly cooperative. Qualitatively similar results were obtained when DGK1 was incubated with vesicles that

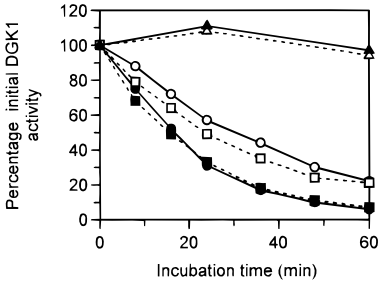

FIGURE 3: Effect of $MgCl_2$ on the heat-induced inactivation of DGK1. The following incubation mixtures containing DGK1 were treated for the indicated periods of time at 45 °C, and then assayed for activity: DGK1 + MOPS−NaCl buffer (closed circles, solid line); DGK1 + MOPS−NaCl buffer + vesicles (closed squares, dotted line); DGK1 + MOPS−NaCl buffer + 1 mM $MgCl_2$ (open circles, solid line); DGK1 + MOPS−NaCl buffer + 1 mM $MgCl_2$ + vesicles (open squares, dotted line). The vesicles contained 52 mol % heart PE + 20 mol % brain PS + 20 mol % egg PC + 8 mol % 18:0−20:4-DG and were present at a concentration of 1 mM total lipid. As controls, incubation mixtures containing DGK1 were treated for the indicated periods of time at 4 °C, and then assayed for activity: DGK1 + MOPS−NaCl buffer (closed triangles, solid line) or DGK1 + MOPS−NaCl buffer + 1 mM $MgCl_2$ (open triangles, dotted line). The percentage of DGK1 activity remaining after the incubation was calculated by dividing the DGK1 activity at each time point by the activity of the same mixture at zero time in order to control for small changes in activity caused by the $MgCl_2$ or the vesicles. Similar results were obtained in a second experiment.

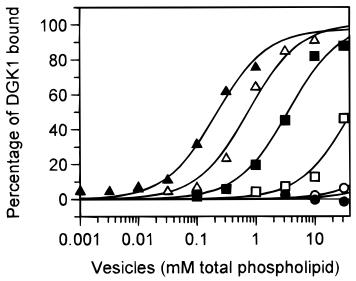

FIGURE 4: Effect of PS on the binding of DGK1 to PC-containing vesicles in the presence of 2 mM $MgCl_2$. DGK1 in MOPS−NaCl buffer + 2 mM $MgCl_2$ was separately incubated with vesicles that were composed of the following mixtures of brain PS and egg PC: 100 mol % PS (closed triangles), 80 mol % PS + 20 mol % PC (open triangles), 60 mol % PS + 40 mol % PC (closed squares), 40 mol % PS + 60 mol % PC (open squares), 20 mol % PS + 80 mol % PC (open circles), 100 mol % PC (closed circles). The vesicles were then pelleted by centrifugation, and DGK1 activities in the supernatant and pellet fractions were measured (Materials and Methods). Recovery of DGK1 activity was >50% in all assays, and the data were expressed as the percentage of vesicle-bound DGK1 activity versus total DGK1 activity. The apparent dissociation constant, K_D^{app}, for each type of vesicle was calculated and used to generate the curves that are shown. Similar results were obtained in two comparable experiments.

consisted of a mixture of egg PC + liver PI, a mixture of egg PC + egg PC-derived PG, or a mixture of egg PC + egg PC-derived PA (Figure 4 and data not shown). However, the effect of egg PC-derived PA was greater than that of brain PS, liver PI, or egg PC-derived PG. Indeed, the K_D^{app} for PA-containing vesicles was lower than that for vesicles that contained twice as much PS (data not shown).

Effects of PE and Phosphoglyceride Fatty Acyl Chain Composition. It was not surprising to find that DGK1 could bind to vesicles that contained anionic phosphoglycerides + PC because it also bound to dextran sulfate−Sepharose (Figure 1). Its binding to the negatively charged vesicles

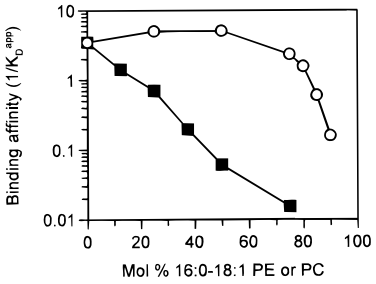

FIGURE 5: Effect of PE on the binding of DGK1 to vesicles. DGK1 was incubated in the presence of 1 mM $MgCl_2$ and vesicles composed of 16:0−18:1-PS + the indicated mol % of 16:0−18:1-PE (open circles) or 16:0−18:1-PC (closed squares). The amounts of bound DGK1 activity were determined as in Figure 4 for three to five different total lipid concentrations; calculated values for enzyme binding affinity ($1/K_D^{app}$) are shown as a function of the mol % of PE or PC in the vesicles. Note that vesicles that contained no PE or PC contained 100 mol % PS. Similar results were obtained in a second experiment.

probably depended on an electrostatic charge effect (*54*). However, the concentration of anionic phosphoglycerides required for effective binding of DGK1 was much higher than that expected to be present in cell membranes. This raised the possibility that additional phosphoglyceride determinants of DGK1 binding might exist. To explore this possibility, we compared the enzyme's ability to bind to vesicles that contained various mixtures of PC + PS with its ability to bind to vesicles that contained corresponding mixtures of PE + PS. Furthermore, we used 16:0−18:1 molecular species of each of the three phosphoglycerides to simplify interpretation of the results. As expected, successive, stepwise increases in the relative content of PC from 10 mol % though 75 mol % were accompanied by stepwise decreases in enzyme binding affinity (Figure 5); the overall decrease in affinity was >1000-fold. In contrast, corresponding increases in the relative content of PE had little effect until the content of PS was reduced to below 25 mol %, when enzyme binding decreased precipitously (Figure 5). These results raised the possibility that PC might be inhibiting the enzyme's binding to vesicles, whereas PE might be acting as a neutral diluent.

We obtained results that were consistent with these headgroup-dependent effects when we compared the ability of DGK1 to bind to vesicles that consisted of 25 mol % 16:0−18:1-PS + 75 mol % 16:0−18:1-PC with its ability to bind to vesicles that consisted of 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 16:0−18:1-PE (Figure 6). The enzyme did not bind detectably to the vesicles that contained PS + PC, as expected. However, it did bind to the vesicles that contained PS + PC + PE (Figure 6).

Experiments with vesicles that contained different proportions of brain PS + egg PC + heart PE yielded qualitatively similar results, but the enzyme appeared to bind more tightly to these vesicles than it did to vesicles that contained comparable proportions of 16:0−18:1-PS + 16:0−18:1-PC + 16:0−18:1-PE (Figure 6). This suggested that phosphoglyceride fatty acyl chain composition also might be an important variable. To investigate this possibility further, we did parallel incubation experiments with DGK1 and vesicles that contained different mixtures of phosphoglyceride molecular species (Figure 6). The results of these experiments revealed that the enzyme bound almost as tightly to vesicles

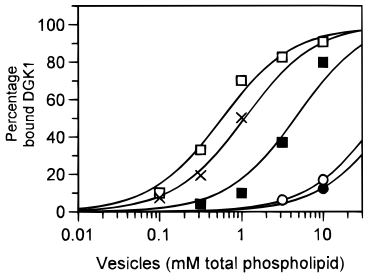

FIGURE 6: Effects of different PE molecular species on binding of DGK1 to vesicles. Aliquots of DGK1 were incubated separately in the presence of 2 mM $MgCl_2$ with vesicles that contained 25 mol % brain PS + 25 mol % egg PC + 50 mol % heart PE (open squares), 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 18:0−20:4-PE (crosses), 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 16:0−18:1-PE (filled squares), 25 mol % brain PS + 75 mol % egg PC (open circles), or 25 mol % 16:0−18:1 PS + 75 mol % 16:0−18:1 PC (filled circles). After the incubation, the vesicles were concentrated by centrifugation, and the bound enzyme was recovered by the Triton X-114 phase separation method and assayed. K_D^{app}, calculated from these data, was used to generate the curves that are shown. Comparable results were obtained in two other experiments.

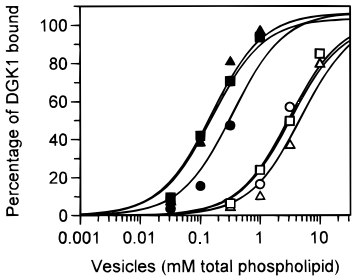

FIGURE 7: Cholesterol and 16:0−18:1-PE have similar effects on DGK1 binding to vesicles. Aliquots of DGK1 were incubated in the presence of 2 mM $MgCl_2$ and vesicles that contained 100 mol % 16:0−18:1-PS (filled circles), 50 mol % 16:0−18:1-PS + 50 mol % 16:0−18:1-PE (filled triangles), 50 mol % 16:0−18:1-PS + 50 mol % cholesterol (filled squares), 50 mol % 16:0−18:1-PS + 50 mol % 16:0−18:1-PC (empty circles), 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 16:0−18:1-PE (empty triangles), or 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % cholesterol (empty squares). After the incubation, the amount of bound enzyme activity was assayed as in Figure 6. Similar results were obtained in two different experiments.

that contained 16:0−18:1-PS + 16:0−18:1-PC + 18:0−20:4-PE as it did to vesicles that contained comparable proportions of brain PS + egg PC + heart PE, and this seemed consistent with the fact that brain PS and egg PC contain relatively high amounts of 18:1 whereas heart PE contains a relatively high amount of 20:4 (data supplied by Avanti, and results not shown). But the enzyme also bound tightly to vesicles that contained 16:0−18:1-PS + 18:0−20:4-PC + 16:0−18:1-PE (data not shown). Thus, both 18:0−20:4-PE and 18:0−20:4-PC influenced enzyme binding, but by mechanisms that were unclear.

Effects of Cholesterol. To determine whether cholesterol also could influence the binding of DGK1 to vesicles, we included it in vesicles that contained 16:0−18:1 molecular species of phosphoglycerides and compared its effects with those of PE. Vesicles that contained 50 mol % PS + 50 mol % cholesterol bound DGK1 much more avidly than did vesicles that contained 50 mol % PS + 50 mol % PC, as indicated by a dramatic difference in the K_D^{app} (Figure 7, compare data indicated by closed squares with those indicated by open circles). In fact, the effect of substituting cholesterol for PC was nearly identical to the effect of substituting PE for PC (Figure 7, data indicated by closed triangles). However, when cholesterol or PE was included in vesicles without being substituted for PC, i.e., *without altering the molar ratio of PC to PS*, neither lipid increased binding significantly (Figure 7). Compare the enzyme's ability to bind to vesicles that contained 25 mol % PS + 25 mol % PC + 50 mol % cholesterol with its ability to bind to vesicles that contained 25 mol % PS + 25 mol % PC + 50 mol % PE. The fact that cholesterol and PE had similar effects on enzyme binding to acidic vesicles and increased enzyme binding only when they were substituted for PC provided strong evidence that they were neutral diluents, whereas PC was a negative effector.

Effects of DG. To determine whether DG could affect the binding of DGK1 to vesicles, we compared the enzyme's ability to bind to vesicles that contained 8 mol % 16:0−18:1-DG (relative to total phosphoglyceride) with its ability to bind to corresponding, DG-free vesicles. The results revealed

Table 2: Effects of DG on DGK1 Binding to Vesicles[a]

vesicle composition	K_D^{app} (mM) without DG	K_D^{app} (mM) with DG	fold-increase in binding
100 mol % PC	>200	>200	nm
50 mol % PC + 50 mol % PE	>200	>200	nm
100 mol % PC + 100 mol % cholesterol	>200	>200	nm
75 mol % PC + 25 mol % PS	>200	2.512	>80
50 mol % PC + 50 mol % PS	8.344	0.087	96
25 mol % PC + 25 mol % PS + 50 mol % PE	7.159	0.068	105
25 mol % PC + 25 mol % PS + 50 mol % PE + 100 mol % cholesterol	3.437	0.049	70
25 mol % PS + 75 mol % PE	0.420	0.014	30

[a] K_D^{app} values were measured for vesicles containing various mixtures of 16:0−18:1 phosphoglycerides and cholesterol in the presence or absence of 16:0−18:1 DG. The contents of phosphoglycerides and cholesterol were expressed relative to the content of total phosphoglyceride. Binding assays were done in MOPS−NaCl buffer that had been supplemented with 2 mM $MgCl_2$. Fold-increase in binding was calculated as the ratio of the K_D^{app} without DG to that with an additional 8 mol % of DG. When no binding was detected even at 30 mM total phosphoglyceride, the K_D^{app} was described as >200 (mM). When no binding was detected with or without DG, the fold-increase was considered not measurable, "nm". Similar results were obtained in an experiment with vesicles that contained egg PC, heart PC, and brain PS.

that inclusion of this amount of DG in phosphoglyceride vesicles markedly decreased the K_D^{app} (Table 2); the 3-fold variation in this response was the same as the experimental error for measurements of K_D^{app}. An effect of DG was even observed in experiments with vesicles that contained PS + PE, but no PC. Moreover, DG's effect did not depend on the fatty acyl chain composition of the DG because experiments with vesicles that contained 8 mol % 18:1−18:1-DG or 8 mol % 18:0−20:4-DG yielded similar results (data not shown).

A major effect of DG on K_D^{app} was also observed with vesicles made from a mixture of 16:0−18:1-DG + 25 mol % PS + 75 mol % PC, though vesicles made from a

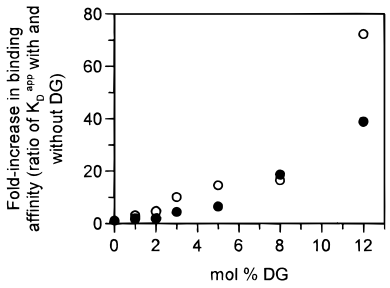

FIGURE 8: Concentration dependence of the DG effect. The binding of DG to vesicles containing various amounts of DG was measured at several concentrations of total lipid. For each mol % DG, the K_D^{app} was calculated, and the ratio of the values for K_D^{app} without and with DG was calculated and termed the fold-increase in binding. Open circles represent vesicles containing 50 mol % 16:0−18:1-PS + 50 mol % 16:0−18:1-PC; filled circles represent vesicles composed of 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 16:0−18:1-PE. Similar results were obtained in two additional experiments.

corresponding mixture of PS and PC that lacked DG failed to bind DGK1. However, vesicles made from mixtures that contained corresponding amounts of DG in addition to (a) 100 mol % PC, (b) 50 mol % PC + 50 mol % PE, or (c) 50 mol % PC + 50 mol % cholesterol failed to bind DGK1 detectably. Thus, the presence of 8 mol % 16:0−18:1-DG in vesicles was not sufficient to induce effective binding.

To examine the concentration dependence of the effect of 16:0−18:1-DG, we added different amounts of this lipid to 1:1 molar mixtures of 16:0−18:1-PS + 16:0−18:1-PC, prepared vesicles from the mixtures, and determined the K_D^{app} for vesicles containing each DG concentration. The results demonstrated that the enzyme's affinity for the vesicles increased as a function of DG content within the range of DG concentrations examined (Figure 8, open circles). Moreover, experiments with vesicles prepared from 2:1:1 molar mixtures of PE, PS, and PC yielded similar results (Figure 8, filled circles). Therefore, the effects of DG on enzyme binding differed from those of PE and cholesterol in two important ways: the *molar* effects of DG were much larger than those of PE and cholesterol, and DG did not have to be substituted for PC.

Effects of Other Potential Substrates and DG Analogues. To examine the molecular basis of the effect of DG, we compared the enzyme's ability to bind to vesicles that had been prepared from mixtures of 20 mol % brain PS + 36 mol % egg PC + 36 mol % heart PE + 8 mol % of either 2-oleoylglycerol, *N*-oleylceramide, or one of several DG analogues. As controls for these experiments, we used vesicles that contained 20 mol % brain PS + 36 mol % egg PC + 44 mol % heart PE. Whereas the presence of 18:1−18:1-DG in vesicles was associated with an increase in enzyme binding of more than 100-fold in these experiments, the presence of 2-oleoylglycerol or *N*-oleylceramide in the vesicles had a negligible effect (Table 3). Furthermore, none of the DG analogues reduced the K_D^{app} as much as did DG (Table 3). Phorbol 12-myristate 13-acetate increased enzyme binding by only 15-fold, dioleoyl-D-glyceramide had a similar effect, and dioleoyl-1-fluoro-2,3-propanediol had only a small effect. The combined results of these experiments suggested that both the hydroxyl group of DG and its diradyl structure might be required for its effect.

Effect of ATP on Binding. ATP also influenced the ability of DGK1 to bind to vesicles, but only in special cases (Figure 9; Table 3). It was without effect on enzyme binding to vesicles that contained neither DG nor DG analogues, and also had little or no effect on enzyme binding to vesicles that contained 8 mol % concentrations of 2-oleoylglycerol, phorbol ester, or dioleoyl-1-fluoro-2,3-propanediol. But it had small, reproducible effects on enzyme binding to vesicles that contained the corresponding amounts of 18:1−18:1-DG or *N*-oleylceramide, and had a large effect on the K_D^{app} (33-fold) observed with vesicles that contained 8 mol % dioleoyl-D-glyceramide. The basis for this last result is unclear, though electrostatic interactions between enzyme-bound ATP and enzyme-bound dioleoyl-D-glyceramide may have been involved.

DISCUSSION

This study focused on the most abundant soluble DGK in Swiss 3T3 cells, a Ca^{2+}-independent enzyme (41) referred to as DGK1. The results of the study provide evidence that the enzyme contains at least two types of binding sites for lipids. One type of binding site interacts with PS or other anionic phosphoglycerides and is most effective when the content of anionic phosphoglycerides is high relative to that of PC, when some of the vesicle phosphoglycerides contain polyunsaturated fatty acids, and when the incubation medium contains 0.5−2.0 mM Mg^{2+}. The second type of binding site interacts with DG and is most effective when the medium contains both Mg^{2+} and ATP. Both types of binding sites must interact with vesicle lipids if optimal enzyme binding to the vesicles is to be achieved.

Inhibitory Effect of PC. The effect of vesicle PC on enzyme binding warrants special comment. The ability of DGK1 to bind to PS-containing vesicles decreased more than 1000-fold when 0−75 mol % PC was substituted for the PS, but underwent little change when 0−75 mol % PE was substituted for the PS in parallel experiments (Figure 5). Moreover, the enzyme's ability to bind to vesicles that contained both PS and PC increased when PE or cholesterol was substituted for the PC, but not when PE or cholesterol was included in the vesicles without changing the ratio of PS to PC (Figure 6). The simplest way to account for these results is to postulate that PC was a strong inhibitor of enzyme binding whereas PE and cholesterol were effectively neutral diluents. An alternate possibility, that PC may have been a neutral component of the vesicles while PE and cholesterol were positive effectors of enzyme binding, appears implausible. The molecular structures of PE and cholesterol have so little in common that separate mechanisms would have to be invoked to account for their potential effects. Furthermore, two specific results of our study are inconsistent with the possibility that PE was a positive effector of enzyme binding. Substitution of >75 mol % PE for PS in vesicles caused a precipitous decrease in enzyme binding (Figure 5), and vesicles that contained 50 mol % heart PE + 50 mol % egg PC did not bind the enzyme detectably (data not shown).

It is of interest that studies of Ca^{2+}-dependent systems by other investigators also have revealed different effects of PC and PE. For example, McLaughlin et al. (*55*) found that the concentration of Ca^{2+} required to induce phosphoglyceride vesicle aggregation was much lower for vesicles that contained either PS alone or a mixture of PS + PE than it

Table 3: Effects of Potential Substrates or DG Analogues on the Binding of DGK1 to Vesicles[a]

potential substrate or analogue	K_D^{app} (mM) with Mg^{2+}	K_D^{app} (mM) with MgATP	fold-increase in binding: substrate or analogue effect with Mg^{2+}	fold-increase in binding: substrate or analogue effect with MgATP	fold-increase in binding: ATP effect
none	19.32 (1.6)	10.75 (1.2)			1.8 (1.4)
2-oleoylglycerol	10.78 (2.3)	6.66 (1.3)	1.8 (1.6)	1.6 (1.6)	1.6 (1.4)
N-oleylceramide 2.8(1.3)	8.08 (1.5)	2.93 (1.9)	2.4 (1.7)	3.7 (2.0)	
18:1−18:1-DG	0.17 (2.1)	0.05 (1.3)	111.2 (1.4)	196.2 (1.0)	3.2 (1.2)
dioleoyl-D-glyceramide	1.26 (1.7)	0.04 (2.3)	15.4 (1.6)	262.1 (2.4)	30.6 (1.1)
phorbol 12-myristate 13-acetate	1.28 (1.5)	0.57 (3.1)	15.1 (1.2)	18.8 (3.2)	2.2 (2.0)
dioleoyl-1-fluoro-2,3-propanediol	2.41	1.20 (1.4)	8.0 (1.5)	9.0 (1.4)	2.0 (1.2)

[a] K_D^{app} values were measured for vesicles composed of 20 mol % brain PS + 36 mol % egg PC + 36 mol % heart PE + 8 mol % potential substrate or DG analogue, as indicated. Vesicles that lacked potential substrates or analogues contained an additional 8 mol % heart PE because this phosphoglyceride had almost no effect on enzyme binding when the ratio of PS to PC was kept constant. Binding assays were done in the presence of 5 mM $MgCl_2$ alone or 5 mM $MgCl_2$ + 0.2 mM ATP. Effects of potential substrates or DG analogues were calculated as ratios of K_D^{app} without the potential substrates or DG analogues to K_D^{app} with the potential substrates or DG analogues. Effects of ATP were similarly calculated as ratios of K_D^{app} without ATP to K_D^{app} with ATP for each potential substrate or DG analogue. Results shown represent means of three different experiments; standard errors (given in parentheses) were calculated using a log transformation and were expressed as multiplicative errors (not additive errors), as described by Fisher et al. (*76*).

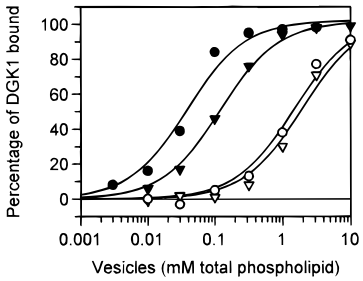

FIGURE 9: Effect of $MgCl_2$ and ATP on association of DGK1 to vesicles containing or not containing DG. Binding of DGK to vesicles was measured as described with (circles) or without (triangles) 0.2 mM ATP added to the incubation mixtures. All mixtures also contained 5 mM $MgCl_2$. The vesicles were composed of 36 mol % egg PC + 36 mol % PE + brain 20 mol % PS, and either 8 mol % 18:1−18:1-DG (filled symbols) or an additional 8 mol % heart PE (open circles). K_D^{app} values, calculated from the data, were used to generate the curves that are shown. Similar results were obtained in two different experiments.

was for vesicles that contained a mixture of PS + PC. Ekerdt and Papahadjoupoulos (*56*) found that large liposomes that contained PS alone or a mixture of 50 mol % PS + 50 mol % PE bound Ca^{2+} with a higher affinity than did liposomes that contained a mixture of 50 mol % PS + 50 mol % PC. These results are of particular interest because they are likely to have involved only interactions with phosphoglyceride headgroups.

Studies of protein−vesicle binding phenomena, which could potentially have involved other mechanisms, also revealed different effects of PC and PE. For example, Bazzi et al. (*57*) found that substitution of PE for PC in large unilamellar, PS-containing vesicles reduced by about 10-fold the concentration of Ca^{2+} required for binding of different annexins. Furthermore, Mosior et al. (*58*) found that substitution of 16:0−18:1-PE for 16:0−18:1-PC in vesicles that contained 30 mol % 16:0−18:1-PS increased the ability of the vesicles to bind protein kinase C in the presence of 0.2 mM Ca^{2+}. Thus, PC appears to have had inhibitory effects on enzyme binding phenomena in these systems as well as in ours.

Potential inhibitory mechanisms that have to be considered include mechanisms related to headgroup size and spacing, fatty acyl chain packing, and phosphoglyceride lateral phase separation phenomena (for recent reviews, see refs *59*−*62*). In addition, dipole−dipole interactions among the headgroups of the phosphoglycerides could conceivably be involved (*63*−*66*). Our current working hypothesis is that a basic, hydrophilic region on the surface of DGK1 interacts electrostatically with the negatively charged surface of a PS-containing vesicle and that the phosphorylcholine headgroups of PC molecules inhibit this interaction via an electrostatic shielding mechanism. Of course, it is also necessary to postulate that the phosphorylethanolamine headgroup of PE does not have a comparable electrostatic shielding effect.

It might be possible to test some aspects of this hypothesis by measuring the binding of hydrophilic model peptides to unilamellar vesicles. Several investigators have studied the interactions of such peptides with vesicles that contain PC and PS, and inhibitory effects of PC have been demonstrated (*62*, *67*). But the effects of vesicle PE on peptide binding have not been examined systematically. If experiments with model peptides were to reveal effects of PE on peptide binding that differ from those of PC, it might be possible to use high-resolution NMR to determine whether the differences correlate with differences in the orientation of elements of the phosphorylserine headgroup of PS.

Effects of DG, DG Analogues, and ATP. DGK1 binding to vesicles increased manyfold when the content of DG in the vesicles was increased from 0 to 12 mol % (Figure 8). The effect of DG probably depended at least in part on an interaction with the enzyme's substrate binding site for DG. In support of this possibility, DG had an even greater effect on enzyme binding when ATP, which had no effect by itself, was present in the medium (Table 3). Furthermore, dioleoyl-D-glyceramide was both a potent effector of enzyme binding in the presence of ATP (Table 3) and an effective competitive inhibitor of DGK1 activity in assays with substrate-containing vesicles (*41*). However, other potential explanations for the effects of DG have to be considered. For example, DG binding sites in addition to the enzyme's substrate binding site may have been involved. All of the DGKs that have been sequenced to date contain "zinc-fingerlike regions" that resemble the DG and phorbol ester binding regions in protein kinase C isoforms (*21*−*23*); and the possibility that similar regions in DGK1 may have promoted the enzyme's binding to vesicles remains to be excluded. It is also conceivable

that the presence of DG in the vesicles may have altered the physical properties of the vesicle surface (see, for example, refs *68*−*72*). Physical effects of DG on enzyme-vesicle interactions appear to have been observed in other systems (*73*−*75*).

In summary, we have provided evidence that DGK1 contains at least two types of binding sites for vesicle lipids. One type of binding site interacts with negatively charged phosphoglycerides and is most effective when the vesicles contain high amounts of these phosphoglycerides relative to PC and the medium contains Mg^{2+}. The second type of binding site interacts with DG and is most effective when the medium contains Mg-ATP. Definitive evidence regarding the properties of these binding sites will have to await studies with a pure preparation of DGK1. In the accompanying paper (*41*), we describe an affinity purification procedure for DGK1 that is based on the results of the present binding study. In addition, we describe vesicle binding experiments with a 3000-fold-purified preparation of DGK1 and explore the relation between enzyme binding and activity in vesicle assay systems. Further work will be needed to characterize the structure of the enzyme and its membrane-associated, apparent counterpart; establish the molecular basis of the effects of vesicle lipids on enzyme binding; and identify mechanisms that influence the timing and direction of enzyme binding to membranes in intact cells.

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