Affinity Purification and Catalytic Properties of a Soluble, Ca^{2+}-Independent, Diacylglycerol Kinase†

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ABSTRACT: We used a new procedure that involved selective enzyme binding to lipid vesicles to partially purify a soluble diacylglycerol kinase, then studied the relation between enzyme-vesicle binding and activity in vesicle-based assays. The vesicle-binding procedure required about 2 h, increased the enzyme's specific activity 50-fold with a 50% yield of activity, and combined well with additional purification steps. Studies of the activity of the partially purified diacylglycerol kinase toward vesicle-associated diacylglycerols revealed linear reaction kinetics that reflected enzyme binding to the vesicles; factors known to influence enzyme binding to the vesicles affected enzyme activity only indirectly, not by influencing the diacylglycerol kinase reaction itself. On the other hand, special incubation experiments that caused both substrate depletion in vesicles and enzyme stalling provided evidence that the diacylglycerol kinase could desorb from these vesicles, adsorb to freshly added, substrate-containing vesicles, and resume catalysis of phosphorylation reactions. The molecular basis for this enzyme−vesicle "hopping" behavior remains to be clarified. But enzyme-catalyzed conversion of diacylglycerol to phosphatidic acid may not have been a contributing factor because separate, enzyme−vesicle binding experiments showed that the enzyme had only a marginally higher affinity for diacylglycerol-containing vesicles than it did for vesicles that contained comparable amounts of phosphatidic acid. The combined results of our experiments suggest that the linear rates of diacylglycerol phosphorylation observed in standard assays with diacylglycerol-containing vesicles may have been combined functions of both the rate of enzyme hopping among vesicles and the rate of diacylglycerol phosphorylation by enzyme that was bound transiently on substrate-containing vesicles.

We have been studying the behavior of a soluble, diacylglycerol kinase from Swiss 3T3 cells to identify factors that might influence the enzyme's ability to bind to and react with membranes in intact cells. We call this diacylglycerol kinase 1 DGK1.[1] In a companion report, we describe factors that influence the ability of DGK1 to bind to unilamellar lipid vesicles (*1*). These factors include PS and other anionic phosphoglycerides, which promote DGK1 binding to membranes, and PC, which seems to inhibit DGK1 binding. Moreover, DGK1 may contain at least two types of binding sites for vesicle lipids: one that interacts with anionic phosphoglyceride and another that interacts with DG. In addition, it appears that the two types of binding sites must act together if optimal DGK1 binding to membranes is to be achieved.

In the present study, we used the information that we had obtained about DGK1 binding to develop a vesicle-based affinity purification method for the enzyme. In addition, we used vesicle-based assays to investigate the enzyme's catalytic properties. In principle, a soluble enzyme that catalyzes reactions that involve an insoluble, membrane-associated lipid must first associate with a membrane surface before it binds its substrate and catalyzes the conversion of the substrate into product. Furthermore, an enzyme that has a high affinity for a membrane surface and dissociates very slowly from that surface may remain bound to the membrane and continue to catalyze reactions on it until the supply of substrate has been exhausted or product-dependent inhibition occurs. For enzymes that catalyze reactions involving vesicle-associated substrates in vitro, this process has been called "scooting" (*2*, *3*). On the other hand, an enzyme that binds to a membrane, and then dissociates quickly from it, may catalyze a few reactions on the membrane, and then dissociate from the membrane's surface and bind to another membrane. This has been called "hopping."

With these possibilities in mind, we addressed several questions with regard to DGK1: (1) What conditions are required for linear reaction kinetics in unilamellar lipid vesicle assay systems? (2) Do vesicle phosphoglycerides that influence DGK1 binding to vesicles have additional effects on the enzyme's activity? (3) Does DGK1 act primarily in the scooting mode or in the hopping mode? (4) What is the molecular basis for this action? Our results demonstrate that vesicle assays can be a powerful tool for the study of DGKs.

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[1] Abbreviations: DG, diacylglycerol; 18:0−20:4-DG, *sn*-1-stearoyl-2-arachidonoyl-DG; 18:1−18:1-DG, *sn*-1-oleoyl-2-oleoyl-DG; DGK1, diacylglycerol kinase 1, the major soluble diacylglycerol kinase in Swiss 3T3 cells; DGKs, diacylglycerol kinases; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; K_D^{app}, apparent dissociation constant; K_M^{app}, apparent K_M; K_I^{app}, apparent K_I; MG, 2-monoacylglycerol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; 16:0−18:1-PS, -PC, or -PE, *sn*-1-palmitoyl-2-oleoyl molecular species of these phosphoglycerides.

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Table 1: Purification of DGK1 from 3T3 Cell Cytosol[a]

fraction	specific activity (pmol min^{-1} mg^{-1})	purification (*x*-fold)	yield (% of total activity)
3T3 cell cytosol	0.2	1	100
dextran sulfate peak 1	1.8	10	27
bound to DG vesicles	100.6	570	13
Superose 12 peak	560.6	3178	4

MATERIALS AND METHODS

Materials. Most materials were as described in the companion paper (*1*). Centricon concentrators were purchased from Amicon, and Superose 12 was purchased from Pharmacia.

Partial Purification of DGK1. The high-speed supernatant fraction from homogenates of quiescent 3T3 cells was prepared, loaded onto a dextran sulfate−Sepharose column, and eluted as described (*1*). Fractions corresponding to DGK1 were pooled and concentrated using a Centricon-30 concentrator, diluted with MOPS−NaCl buffer (50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 0.1 mM DTPA), and then concentrated again, so that the final concentrate contained less than 5% of the original buffer and half the volume. DGK1 activity in the concentrate was purified further by differential binding to unilamellar lipid vesicles in two successive steps as follows. First, 12 mL of DGK1 concentrate containing 2.6 mg/mL total protein was incubated for 2 min at 37 °C with a mixture containing (a) 480 μL of 50 mM test vesicles composed of 25 mol % 16:0−18:1-PS + 50 mol % heart PE + 25 mol % 16:0−18:1-PC + 1 mol % added, biotinylated PE and, (b) to speed up precipitation, 720 μL of "carrier" vesicles composed of 99 mol % egg PC + 1 mol % biotinylated PE. After the addition of avidin (1200 μL of a 15 mg/mL solution), the mixture was incubated for 5 min at 37 °C to allow the vesicles to precipitate, and the precipitate was pelleted by centrifugation for 10 min at 16000*g*. Second, the supernatant from the centrifugation step, which contained most of the DGK1, was incubated in a similar manner but with vesicles that were composed of 10 mol % 16:0−18:1-DG + 25 mol % 16:0−18:1-PS + 50 mol % heart PE + 15 mol % 16:0−18:1-PC + 1 mol % added, biotinylated PE at a final vesicle concentration of 2 mM total lipid. Avidin was added, and the mixture was incubated and centrifuged as before, but this time essentially all of the DGK1 was recovered in the pellet fraction. The pellet was resuspended once in MOPS−NaCl buffer and centrifuged a second time to remove unbound or weakly bound protein. The final, washed pellet was solubilized in 6% Triton X-114 at 4 °C and separated into aqueous and detergent phases by precipitation of the Triton at 37 °C (*4*).

After this, all steps were performed at 4 °C. The upper aqueous phase containing the DGK1 was concentrated in a Centricon-50 concentrator to a volume of 105 μL, and 100 μL of the concentrate was chromatographed on a column of Superose 12 in buffer containing 0.2 M phosphate, pH 6.7, and 0.05% reduced Triton X-114. Fractions that contained the bulk of the DGK1 activity were pooled and used for most of the experiments described in this paper. Table 1 describes the results of the purification.

Vesicle Methods. Unilamellar, 100-nm diameter lipid vesicles of various compositions were made by extrusion as described previously (*1*). All vesicles used were stable for at least 1 week. Optical densities of suspensions of the vesicles did not differ significantly from that of an equal concentration of vesicles of known, 100-nm diameter. Binding of DGK1 to the vesicles was determined using an avidin precipitation method (*5*) as described (*1*) The apparent dissociation constant (K_D^{app}) for a given vesicle composition was calculated from the fractions of DGK1 bound and unbound at three or more concentrations of the vesicles, using a mathematical fit as described (*1*).

DGK1 Activity Measurements. DGK1 activity assays contained 0.2 mM of 100−2000 cpm/pmol [γ-^{32}P]ATP + 2 mM $MgCl_2$ + vesicles of the composition and concentration indicated in each experiment + 0.1−2 μL (1.5−30 μg) of approximately 3000-fold purified DGK1 (from the Superose-12 column) in MOPS−NaCl buffer at a total volume of 50 μL, unless otherwise indicated. Except where noted specifically, standard assay vesicles composed of 52 mol % heart PE + 8 mol % 18:0−20:4-DG + 20 mol % brain PS + 20 mol % egg PC were used, at a total lipid concentration of 10 mM. The reaction mixtures were incubated for 10 min at 37 °C, and then quenched and processed for counting of radioactive PA as described (*1*). Under these conditions, the formation of PA was linear with enzyme (not shown) and time (Figure 3) and was saturating for all other components (see Figures 4 and 6).

Other Methods. Protein concentrations were determined by the method of Bradford (*6*). Phosphoglyceride concentrations were determined by phosphate assays following acid hydrolysis (*7*), and DG, MG, and dioleoyl-D-glyceramide concentrations were determined by ester assays (*8*). SDS−PAGE on minigels (Bio-Rad) was performed by the method of Laemmli (*9*).

RESULTS

Partial Purification of DGK1 by Selective Adsorption on Vesicles. In our study of factors that influence the ability of DGK1 to bind to unilamellar, phosphoglyceride-containing vesicles (*1*), we found that under appropriate incubation conditions some vesicles bound the enzyme only when they contained DG. This suggested that these vesicles could be used for affinity purification of the DGK1. To investigate this possibility, we incubated DGK1 (which had been partially purified by chromatography on dextran sulfate−Sepharose) with DG-free vesicles or DG-containing vesicles, and then measured the amounts of bound protein and enzyme activity that could be recovered. Only small amounts of protein bound to the DG-free vesicles (Figure 1A) and the DG-containing vesicles (Figure 1B), but most of the DGK1 *activity* bound selectively to the DG-containing vesicles. Furthermore, a two-step procedure that took advantage of this selectivity could be used to achieve an even greater purification (Figure 1C). First, DGK1 was incubated with DG-free vesicles to remove proteins that could bind to the vesicle phosphoglycerides. Then, the unbound fraction from this step was incubated with DG-containing vesicles that had the same relative phosphoglyceride composition. As shown in the figure, even less total protein was measured in the final bound fraction with this method than with the one-step method. Thus, this procedure capitalized on both the general requirements and the specific DG dependence for DGK1 binding to membranes. A variation of the two-step

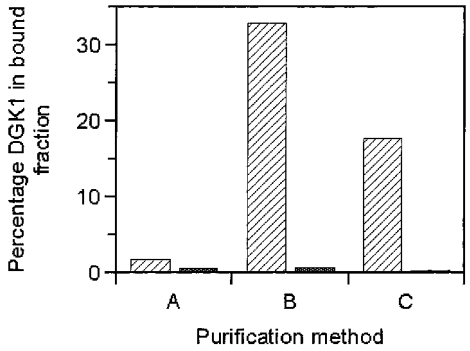

FIGURE 1: Selective adsorption of DGK1 to unilamellar lipid vesicles. DGK1 that had been purified about 10-fold by chromatography on a column of dextran sulfate−Sepharose (1) was incubated with unilamellar, phosphoglyceride-containing vesicles in three ways in order to identify a method for further purifying the DGK1. (A) It was incubated with DG-free vesicles that contained a mixture of 25 mol % 16:0−18:1-PS + 25 mol % 16:0−18:1-PC + 50 mol % 16:0−18:1-PE in addition to 1 mol % biotin-PE relative to total phosphoglyceride. (B) It was incubated with vesicles that had the same relative composition of phosphoglycerides and biotin−PE but contained 8 mol % of added DG. (C) It was incubated with DG-free vesicles of the type used in (A); then the unbound material from the incubation was incubated with the DG-containing vesicles of the type used in (B). Each of the incubations contained 5 mM total vesicle lipid + 2 mM $MgCl_2$ in MOPS−NaCl buffer, and each was done for 2 min at 37 °C. Avidin at a concentration of 1.25 mg/mL was added immediately afterward, and the incubation was continued for an additional 10 min. The precipitated vesicles were centrifuged for 10 min at 16000*g*, resuspended, and washed once with buffer to remove loosely bound protein. The remaining bound protein was then separated from the lipid and lipid-bound avidin using the Triton X-114 phase separation method of Bordier (4). The aqueous phase of the Triton separation was processed for measurement of DGK1 (hatched bars) and total protein (closed bars) as described under Materials and Methods. Similar results were obtained in a second experiment and in additional experiments where only enzyme activity was measured.

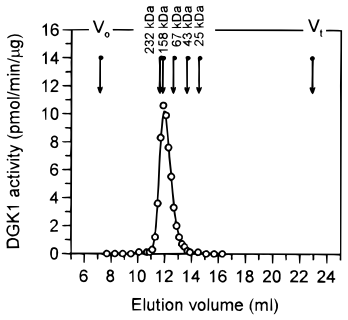

FIGURE 2: Size-exclusion chromatography of affinity-purified DGK1. DGK1 that had been purified by selective, stepwise adsorption on vesicles as shown in Figure 1 was concentrated in a Centricon-50 concentrator. Then 100 μL of the concentrate was chromatographed on a column of Superose 12 in 0.2 M phosphate, pH 6.7, containing 0.05% reduced Triton X-114. DGK1 activity was assayed in the column fractions and is shown as a function of elution volume. The arrows in the figure indicate the elution volumes of blue dextran 2000 (the void volume, V_0), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and silver nitrate (the total column volume, V_t). DGK1 eluted as shown in two of three experiments with affinity-purified DGK1 and in two trial runs with less pure enzyme, but was delayed by 0.8 mL in the third experiment with affinity-purified DGK1.

method, which yielded a 57-fold purification of DGK1, was used in the final enzyme purification procedure (see Materials and Methods).

DGK1 that had been purified by selective adsorption on vesicles was concentrated and loaded onto a Superose 12 size-exclusion column for further purification. Column preequilibration and chromatography were done in the presence of 0.05% reduced Triton X-114 because trial experiments with less pure DGK1 had shown that this diminished enzyme loss; 47% of the DGK1 activity was recovered from the column in the presence of Triton X-114, whereas only 9% was recovered in the absence of Triton X-114. The elution volumes of DGK1 activity and the standard proteins were identical in the presence or absence of Triton X-114 (not shown). In the presence of Triton X-114, the size-exclusion procedure yielded a single, symmetrical peak of DGK1 activity, which provided evidence that only one DGK was present (Figure 2). Furthermore, analysis of pooled material from the DGK1 peak demonstrated a 6-fold increase in the enzyme's specific activity and an overall enzyme purification of about 3000-fold (Table 1).

The molecular mass of DGK1 remains uncertain. In four of five experiments performed using the Superose 12 column, DGK1 had an apparent molecular mass of 150 kDa, as determined from the elution volumes of standard proteins (Figure 2). But in one experiment DGK1 eluted 0.8 mL later, at a volume corresponding to that of the 67 kDa standard. The cause of this variability was not determined, but for the latter experiment the column had been prewashed with a cleaning solution to remove any residual protein, so DGK1 may have interacted slightly with the unblocked column matrix. Analysis of the 3000-fold purified enzyme preparation by SDS−PAGE did not resolve this issue because it yielded five visible bands (not shown). Therefore, the DGK1 was still not pure, but the likelihood that the fraction contained proteins that would interfere with subsequent assays of enzyme activity was reduced.

DGK1 Activity toward Substrates in Vesicles. We measured the activity of the 3000-fold purified DGK1 toward substrates that were included in "standard assay vesicles" composed of 20 mol % brain PS + 20 mol % egg PC + 52 mol % heart PE + 8 mol % substrate. The enzyme bound to these vesicles with a high affinity. Enzyme activity toward 18:0−20:4 DG in these vesicles was at least as high as the activity seen in octyl glucoside/PS mixed micellar assays (data not shown). When the assays contained 10 mM total lipid, 0.2 mM ATP, and 2 mM $MgCl_2$, the formation of PA was linear with enzyme (not shown) and also was linear with time through at least 1 h (Figure 3). A concentration of 2 mM $MgCl_2$ was saturating (Figure 4A), and the K_M^{app} for ATP was 0.03 mM (Figure 4B). Addition of 100 μM $CaCl_2$ to the 2 mM $MgCl_2$ in the incubation medium had no further effect (data not shown), so the DGK1 activity was Ca^{2+}-independent. The maximum activity measured for 18:0−20:4 DG was 1740 pmol min^{-1} (g of protein)$^{-1}$, the K_M^{app} was 0.5 mol % (Figure 4C), and the enzyme showed little specificity for DG molecular species (not shown).

We also tested the enzyme's activity toward vesicles that contained 2-oleoylglycerol or *N*-oleylceramide because we had examined the effects of these potential substrates in our companion study of enzyme binding (*1*). DGK1 catalyzed the phosphorylation of both compounds, but at maximum rates that were lower and with K_M^{app} that were at least 10-fold higher than the corresponding values for DG-containing

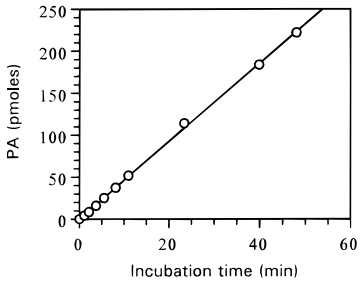

FIGURE 3: Time course of DGK1 reaction. Three-thousand-fold purified DGK1 (7 ng of protein/10 μL of assay) was incubated in MOPS−NaCl buffer with standard assay vesicles (20% brain PS + 20% egg PC + 52% heart PE + 8% 18:0−20:4-DG) at a concentration of 10 mM total lipid in the presence of 0.2 mM [^{32}P]-ATP and 2 mM $MgCl_2$. The assay was quenched and PA isolated at the times indicated. Comparable results were observed in a second experiment with similarly purified enzyme and in two other experiments with a 10-fold purified DGK1 preparation.

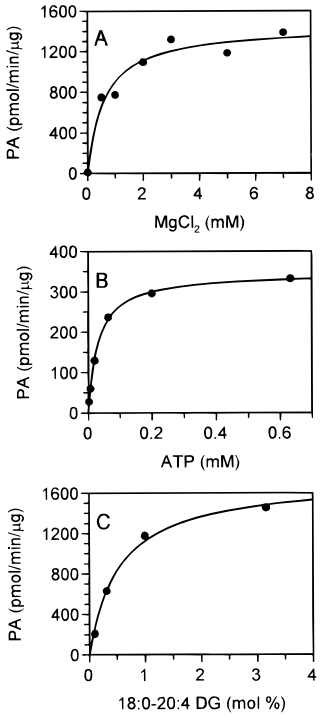

FIGURE 4: Dependence of DGK1 activity on concentrations of medium $MgCl_2$ and ATP and vesicle DG in vesicle assays. The dependence of PA formation on $MgCl_2$ (A), ATP (B), and DG (C) is shown. Except where the component was varied as indicated, assays contained 3000-fold purified DGK1 (1.4 *ng*/10 L assay), 5 mM standard assay vesicles, 0.2 mM [γ-^{32}P]ATP (400−1000 cpm/pmol), and 2 mM $MgCl_2$. Essentially the same results were seen in two duplicate experiments using 10-fold purified DGK1.

vesicles (Figure 5A). The maximum rate of activity for 2-oleoylglycerol was not measurable, whereas that for *N*-oleylceramide was 34 pmol min^{-1} g^{-1} and that for 18:1−18:1-DG was 356 pmol min^{-1} g^{-1}. The K_M^{app} for 2-oleoylglycerol was too high to measure accurately (above 30 mol %), that for *N*-oleylceramide was 5.5 ± 0.7 mol %, and that for 18:1−18:1-DG was 0.60 ± 0.3 mol %. These low values for DGK1 activity toward 2-oleoylglycerol and *N*-oleylceramide are consistent with our observation that these compounds had negligible effects on the enzyme's binding to vesicles (*1*).

DG Analogues Inhibit DGK1 Activity. We also tested the effects of several DG analogues on the DGK1-catalyzed

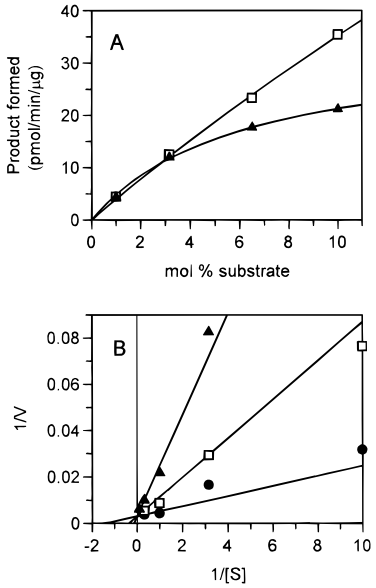

FIGURE 5: DGK1 activities toward 2-oleoylglycerol and *N*-oleylceramide, and inhibitory effects of DG analogues on the DGK1-catalyzed phosphorylation of DG. (A) The activity of 3000-fold purified DGK1 toward 2-oleoylglycerol (squares) and *N*-oleoylceramide (triangles). The activities were measured by incubating DGK1 with vesicles that contained 20% brain PS + 20% egg PC + up to 60% heart PE + the indicated percentage of substrate, at a total lipid concentration of 10 mM. DGK1 binds to vesicles at these concentrations even in the absence of substrate (*1*), so the results are independent of the enzyme's binding affinity. The curves were charted using the following values: for 2-oleoylglycerol, K_M^{app} = 72 mol % and apparent V_{MAX} = 288 pmol min^{-1} μg^{-1}; for *N*-oleylceramide, K_M^{app} = 6.1 mol % and apparent V_{MAX} = 34 pmol min^{-1} μg^{-1}. (B) Inhibition of DG phosphorylation by dioleoyl-D-glyceramide is shown in a Lineweaver−Burk plot format. Three-thousand-fold purified DGK1 was incubated with vesicles composed of 20% brain PS + 20% egg PC + up to 60% heart PE + 18:1−18:1 DG as indicated + either 0 (circles), 1% (squares), or 3.2% (triangles) dioleoyl-D-glyceramide. The data represent the means of duplicates; the curves shown are best-fit curves for competitive inhibition and reflect a calculated K_M^{app} for dioleoylglycerol of 0.72 mol %, and a K_I^{app} for dioleoyl-D-glyceramide of 0.35 mol %. For both parts A and B, comparable results were seen in a second experiment with a similar preparation of DGK1 and in two experiments with 10-fold purified DGK1.

phosphorylation of 18:1−18:1-DG in vesicles. One analogue, dioleoyl-D-glyceramide, proved to be an effective competitive inhibitor with a K_I^{app} of 0.31± 0.04 mol % (Figure 5B), comparable with the above-mentioned K_M^{app} for 18:1−18:1-DG. This suggests that dioleoyl-D-glyceramide and 18:1−18:1-DG may have had similar affinities for the active site of DGK1, which is noteworthy because dioleoyl-D-glyceramide and 18:1−18:1-DG had similar effects on DGK1 binding to vesicles in the presence of MgATP (*1*). The large effect of dioleoyl-D-glyceramide on enzyme binding was unlikely to have influenced the kinetic measurements shown in Figure 5 because the incubations were done with concentrations of vesicles that were saturating for enzyme binding. In separate incubation experiments with vesicles that contained no analogue but were otherwise identical to those used in Figure 5, essentially all of the DGK1 bound at 10 mM total lipid (not shown).

Phorbol 12-myristate 13-acetate, which was a moderate stimulator of enzyme binding to vesicles (*1*), was also a moderate inhibitor of the phosphorylation of 18:1−18:1-DG, but the kinetics did not suggest a simple mechanism (not

shown). Dioleoyl-1-fluoro-2,3-propanediol, which was a weak stimulator of enzyme binding, was also a weak inhibitor of DG phosphorylation by an equally unclear mechanism. Thus, the results obtained with these two analogues were not very informative.

Relation between DGK1 Binding to Vesicles and DGK1 Activity. As mentioned earlier, DGK1 and other soluble cytosolic enzymes that catalyze reactions involving membrane-associated lipids must bind to the cytoplasmic surfaces of membranes before they interact with their substrates and catalyze the conversion of the substrates into products (*3*). Furthermore, separate factors may influence each of these steps. In the case of DGK1, we showed that a number of factors could influence enzyme binding to vesicles including the lipid composition of the vesicles, the concentration of the vesicles, and the content of Mg^{2+} and ATP in the incubation medium (*1*). For example, enzyme binding was of highest affinity when the vesicles contained DG plus a high amount of PS (or some other anionic phosphoglyceride) relative to PC. Insofar as changes in lipid composition increase the amount of enzyme bound to vesicles under specific incubation conditions, they would be expected to promote the phosphorylation of DG in parallel, vesicle-based assays.

To explore the possibility that changes in lipid composition might affect DGK1 activity in vesicle-based assays, *even when all of the enzyme is bound*, we compared the rates of DG phosphorylation in separate assays with standard vesicles and vesicles that had other lipid compositions. As expected, the concentration of total lipid required for half-maximal activity of the standard assay vesicles (5 μM total lipid) was much lower than that required for vesicles that contained a higher mole fraction of PC (Figure 6). But for both types of vesicles, the concentration of total lipid required for activity paralleled that required for DGK1 binding to the vesicles (Figure 6). Furthermore, at vesicle lipid concentrations that yielded complete enzyme binding, essentially the same activity was observed for both kinds of vesicles. These observations support three related conclusions. First, DGK1 must bind to vesicles before it can catalyze the phosphorylation of vesicle-associated DG. Second, and as demonstrated previously (*1*), the PC in vesicles can influence DGK1 activity by affecting enzyme binding. Third, when DGK1 is fully bound to vesicles, the PC of the vesicles has no additional effect on the enzyme's activity.

PC appears to affect DGK1 binding to vesicles by inhibiting the enzyme's interaction(s) with PS (*1*). But PS is not absolutely required for DGK1 binding to vesicles because we were able to measure enzyme binding to vesicles that contained 46 mol % heart PE + 46 mol % egg PC + 8 mol % 18:0−20:4-DG but no acidic phosphoglycerides (data not shown). These vesicles bound DGK1 only slightly and therefore had to be used in very high concentrations. Parallel assays with the vesicles revealed that the enzyme's activity was proportional to enzyme binding (data not shown). This result provides additional support for the conclusion that the content of phosphoglyceride in vesicles influences DGK1 activity by affecting enzyme binding but has no further effect on the ability of the fully bound enzyme to interact with its substrates.

Evidence for Enzyme Hopping. When DGK1 binds to the surface of a DG-containing vesicle, does it remain bound to the same vesicle and scoot on its surface until it has catalyzed the phosphorylation of all of the DG, or does it catalyze a few phosphorylation reactions, and then hop from the vesicle and bind to and catalyze reactions involving other vesicles? To address this question, we incubated DGK1 with standard assay vesicles (0.2 mM total lipid) and used a relative amount of DGK1 that would have been expected to bind effectively to the vesicles and deplete the content of total, vesicle-associated DG within the time frame of the experiment if the rate of phosphorylation of the DG had been linear. But the rate of phosphorylation of DG slowed and the reaction essentially stopped by 40 min for reasons that remain to be explained (Figure 7). We then added to the incubation mixture either (a) fresh DGK1, (b) vesicles that contained only PC + DG (to which DGK1 does not bind; ref *1*), or (c) fresh standard assay vesicles, and continued the incubation. The results of the experiment revealed that neither the fresh, added DGK1 nor the added vesicles that contained only PC + DG revived the activity. However, the fresh, added standard assay vesicles did cause the activity to resume, almost at the original initial rate. Vesicle aggregation could not have explained this result, because none was observed in experiments with standard assay vesicles at any concentration in the presence of 0.2 mM $MgCl_2$. Moreover, DG did not appear to transfer between vesicles because the addition of vesicles that contained PC + DG did not revive activity. Therefore, the enzyme appeared to be able to hop from substrate-depleted vesicles to fresh vesicles.

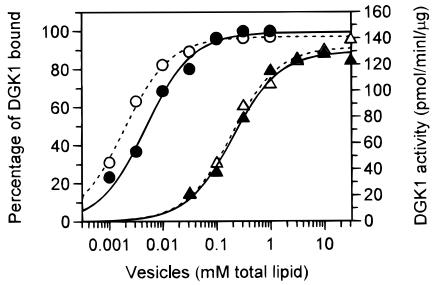

FIGURE 6: Dependence of DGK1 activity and binding on vesicle concentration of DGK1 activity and binding. Both the activity (closed symbols) and the binding (open symbols) of DGK1 toward vesicles were measured over a range of vesicle concentrations. The activity assays contained 3000-fold purified DGK1 (1.5 ng of protein/10 μL of assay), 0.2 mM [γ-^{32}P]ATP, 2 mM $MgCl_2$, and vesicles that were composed of (a) 20 mol % brain PS + 20 mol % egg PC + 52 mol % heart PE + 8 mol % 18:0−20:4-DG + 1 mol % biotinylated PE (circles) or (b) 20 mol % brain PS + 72 mol % egg PC, + 8 mol % 18:0−20:4 DG + 1 mol % biotinylated PE (triangles). The concentration of vesicles required for half-maximal activity was calculated from the data for each vesicle type and used to generate the solid curves shown. The binding incubations contained identical components except that the ATP was not radioactive, and avidin and vesicles composed of 99 mol % PC + 1 mol % biotinylated PE were added after the incubations to precipitate the vesicles. The precipitated vesicles were concentrated by centrifugation, and bound enzyme was recovered by the Triton X-114 phase separation method and assayed (Materials and Methods). Unbound enzyme was also measured, and the percentage of DGK1 bound was determined from the combined data. K_D^{app} values were calculated from the data and used to generate the curves that are shown. Similar results were seen in numerous similar experiments with 10-fold purified DGK1; the binding and activity curves were always within 3-fold of each other for the same vesicle composition, which was within the error observed for the binding experiments described in the companion report (1).

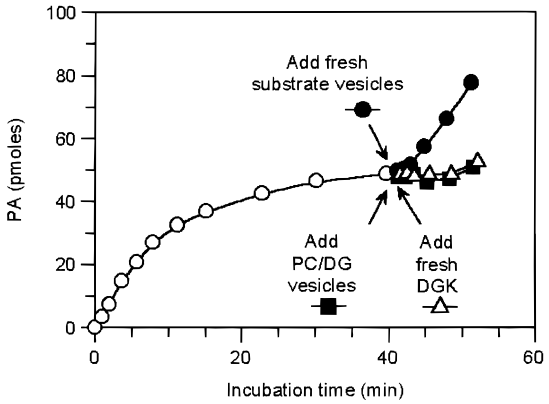

FIGURE 7: DGK1 can hop between vesicles.Three-thousand-fold purified DGK1 (15 ng of protein/10 μL of assay) was incubated with standard assay vesicles (0.2 mM total lipid) at 37 °C. At the times indicated, 10-μL aliquots of the incubation mixtures were removed and processed to measure the amount of PA that had been formed (open circles). After 40 min of incubation, when the DGK reaction had stalled and about one-third of the DG in the vesicles had been converted to PA, an equivalent amount of fresh vesicles was added to the incubation mixture and the incubation was continued (closed circles). Alternatively, the incubation was continued after the addition of vesicles composed of 92 mol % egg PC + 8 mol % 18:0−20:4-DG (closed squares) or after the addition of fresh DGK1 (open triangles). Similar results were obtained in a second experiment, which showed reaction stalling when about 50% of the DG had been converted to PA.

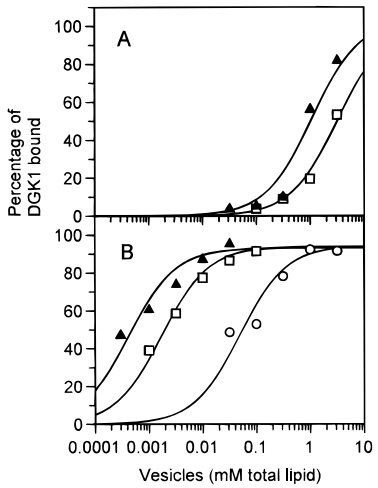

FIGURE 8: Comparisons of the effects of DG and PA on DGK1 binding to vesicles. (A) Three-thousandfold purified DGK1 (7 ng of protein/10 μL of assay) was incubated in the presence of 2 mM $MgCl_2$, 0.2 mM ATP, and vesicles (total lipid as indicated in the figure) that were composed of 20 mol % brain PS + 72 mol % egg PC + either 8 mol % 18:0−20:4-PA (squares) or 8 mol % 18:0−20:4-DG (triangles). Note that DGK1 binding to vesicles composed of similar proportions of PS and PC but no DG or PA was found previously to be unmeasurable (1). After the incubation, the percentage of DGK bound and the K_D^{app} were determined as described in Figure 5. (B) DGK1 was incubated as in (A) except that 0.75 ng of DGK1 protein/10 μL of assay was used and the vesicles were composed of 20 mol % brain PS + 20 mol % egg PC + 52 mol % heart PE + either 8 mol % 18:0−20:4 PA (squares), 8 mol % 18:0−20:4-DG (triangles), or 8 mol % of additional heart PE (circles). We calculated that the activity of the DGK1 in (A) was sufficient to deplete 35% of the available substrate at 0.003 mM total lipid during the 5-min binding incubation, assuming that half of the DGK1 was bound. In (B), we calculated that less than 1% of the DG could have been phosphorylated at 1 mM total lipid with the same assumption. Similar results were obtained in a second experiment; i.e., enzyme binding to vesicles that contained 8 mol % DG was consistently somewhat tighter than it was to vesicles that contained 8 mol % PA.

The molecular basis for this hopping behavior was unclear. But the possibility had to be considered that changes in the enzyme's affinity for vesicles, caused by the enzyme-dependent conversion of vesicle DG to PA, might have been a contributing factor. We had shown that inclusion of DG in vesicles could increase the enzyme's affinity for the vesicles by as much as 2 orders of magnitude and had shown in separate experiments that PA also could increase the enzyme's affinity for vesicles (*1*). But we had not compared the effects of the two lipids in the same experiment so we could not exclude the possibility that the DGK reaction might have had a major effect on enzyme binding in the enzyme hopping experiments. To examine this possibility, we compared the effects of DG and PA in several independent experiments. The results revealed that DGK1 did bind more tightly to DG-containing vesicles than to vesicles that contained comparable amounts of PA, but the differences between the effects of the two lipids were small (Figure 8). This suggests that enzyme hopping may have been essentially independent of the relative amounts of substrate and product in the vesicles.

DISCUSSION

In this study we first developed an affinity purification method for DGK1 that was based on the enzyme's ability to bind selectively to unilamellar lipid vesicles that contained DG, and then characterized the catalytic properties of the purified DGK1 in assays with unilamellar lipid vesicles. The affinity purification method was rapid and effective, and it resulted in high yields of active enzyme. This suggests that the method could be of use in the purification of other soluble DGKs and proteins that bind DG. When the method was combined with two other methods, a 3000-fold overall purification of DGK1 was achieved. The resulting DGK1 preparation was not homogeneous, but its use in subsequent enzyme activity assays greatly reduced the possibility that other proteins in the incubation mixtures could have affected the results.

Our study of the enzyme's catalytic properties yielded much useful information. We found that the enzyme catalyzed the phosphorylation of vesicle-associated DG without showing a preference for different molecular species of DG and that the accumulation of PA was linear with enzyme and time. The enzyme also catalyzed the phosphorylation of vesicle-associated 2-oleoylglycerol and vesicle-associated *N*-oleylceramide but at much lower rates than it catalyzed the phosphorylation of DG. Addition of 100 μM Ca^{2+} had no effect on the enzyme's activity when incubation media already contained 2 mM Mg^{2+}.

However, our findings with regard to the relation between enzyme binding to vesicles and enzyme activity seemed particularly noteworthy. As mentioned earlier, we had identified several factors, including vesicle anionic phosphoglycerides that could influence the ability of DGK1 to bind to vesicle surfaces (*1*). Therefore, we did a series of experiments to examine the effect of vesicle phosphoglyceride composition on the enzyme's activity. We had two possibilities in mind. First, changes in the composition of vesicle phosphoglycerides might influence the phosphorylation of DG *solely* by changing the enzyme's ability to bind

to DG-containing vesicles. Alternatively, changes in vesicle phosphoglycerides might influence not only the ability of soluble DGK1 to bind to vesicles, but also the ability of DGK1 that had already bound to vesicles to catalyze the phosphorylation of DG. Our results clearly support the first possibility because vesicle phosphoglyceride composition had no apparent effect on the measured rate of DG phosphorylation under conditions where essentially all of the enzyme was bound (Figure 5).

When we incubated DGK1 with vesicles until the phosphorylation of DG stalled, less than 50% of the DG was utilized, raising the possibility that net transfer of DG from the inner to the outer bilayer did not occur. When we then added fresh vesicles to the incubation mixture and continued the incubation, DG phosphorylation resumed (Figure 7). This showed that DGK1 could hop from substrate-depleted vesicles to fresh, substrate-containing vesicles and raised the possibility that the linear kinetics that we had observed in our standard assays (Figure 3) might have depended on enzyme hopping. In other words, DGK1 might have bound to a vesicle, catalyzed some DG phosphorylation reactions on that vesicle, and then, well before the reaction stalled, hopped to a new vesicle and catalyzed phosphorylation reactions on it. This would have allowed the enzyme eventually to access all of the vesicles in the assay.

For this model of enzyme action to be valid, two important conditions would have to be satisfied. First, the rate of enzyme dissociation from vesicles would have to be slower than the rate of catalysis by bound enzyme (to allow some substrate to be converted into product), but not so slow that the enzyme reaction stalls. Second, the rate of enzyme reassociation with vesicles would have to be rapid relative to the rate of enzyme dissociation from vesicles (so that the enzyme does not spend significant time in solution). We have yet to measure these rates directly; this will have to await studies with a fully purified enzyme. But the results that we have obtained so far seem consistent with both possibilities. For example, most of our studies of enzyme activity were done with vesicles that showed a low K_D^{app} (the ratio of the rate of enzyme dissociation to that of enzyme reassociation) and at vesicle concentrations well above the K_D^{app} (ref *1* and Figure 6, present study). Therefore, DGK1 reassociated quickly and did not spend significant time in solution. Furthermore, the results shown in Figure 7 provide strong evidence that DGK1 can dissociate rapidly from product-containing vesicles and begin almost immediately to catalyze phosphorylation reactions on fresh, substrate-containing vesicles. Altogether, it appears that a combination of enzyme scooting and hopping behaviors may have occurred in our assays with unilamellar lipid vesicles and allowed linear reaction kinetics. This would imply that the measured rates of DG phosphorylation in the assays reflected both the rates of enzyme hopping and the rates of DG phosphorylation by bound enzyme.

A combination of scooting and hopping behaviors also may have contributed to catalysis by other interfacial enzymes (*10*). On the other hand, most investigations that have been done to date have measured the catalytic rates of lipid-metabolizing enzymes that are exclusively in the scooting mode (*2*, *11*). This has been a useful approach because enzymes remain bound to membranes in that mode, and therefore effects of incubation conditions on enzyme activity can be analyzed independently of enzyme adsorption and desorption phenomena.

In conclusion, we have examined the ability of DGK1 to bind to and react with unilamellar vesicles (ref *1* and present study). Our results raise the possibility that catalysis by DGK1 in assays with these vesicles may involve a combination of scooting and hopping phenomena. Important questions remain to be answered both about the molecular basis for the effects that we have observed and about the relevance of the effects to events that actually occur in vivo. Answers to these questions will have to await final purification of the enzyme, analysis of its molecular structure, and experimentation with intact cells.

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