Effects of Phospholipid Headgroup and Phase on the Activity of Diacylglycerol Kinase of *Escherichia coli*†

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ABSTRACT: Diacylglycerol kinase (DGK) of *Escherichia coli* has been reconstituted into a variety of phospholipid bilayers and its activity determined as a function of lipid headgroup structure and phase preference. The anionic phospholipids dioleoylphosphatidic acid, dioleoylphosphatidylserine, and cardiolipin were all found to support activities lower than that supported by dioleoylphosphatidylcholine. In mixtures of dioleoylphosphatidylcholine and 20 mol % anionic phospholipids, the presence of anionic phospholipids all resulted in lower activities than in dioleoylphosphatidylcholine, except for dioleoylphosphatidylglycerol whose presence had little effect on activity. In some cases, the low activity in the presence of anionic phospholipid followed from a decrease in v_{max}; in some cases, it followed from an increase in the K_m for diacylglycerol, and in the case of dioleoylphosphatidic acid, it followed from both. Activities in mixtures containing 80 mol % dioleoylphosphatidylethanolamine were lower than in dioleoylphosphatidylcholine at temperatures where both lipids adopted a bilayer phase; at higher temperatures where dioleoylphosphatidylethanolamine preferred a hexagonal H_{II} phase, the differences in activity were greater. These experiments suggest that the presence of lipids preferring a hexagonal H_{II} phase leads to low activities. Activities of DGK are low in a gel phase lipid.

Biological membranes contain a complex mixture of lipids, differing in their polar headgroups and fatty acyl chains. All membranes contain both anionic and zwitterionic lipids. The presence of anionic lipids is obviously important for the binding of peripheral membrane proteins to the surface of the membrane. Some peripheral proteins bind through domains specific for a particular class of lipid headgroup such as the PH domain that recognizes phosphoinositides such as PtdIns(4,5)P_2 (*1*). Other peripheral proteins contain extended patches of positively charged residues that interact electrostatically with any anionic lipids in the membrane (*2*). The presence of these anionic phospholipids, of course, has to be compatible with the proper function of the intrinsic membrane proteins present in the membrane. In fact, some intrinsic membrane proteins show a specific requirement for the presence of a particular class of anionic lipid. For example, there is considerable evidence that the presence of cardiolipin is required for the proper function of a number of enzymes involved in oxidative phosphorylation, including cytochrome oxidase (*3*, *4*). Surprisingly, although a tightly bound lipid molecule has been identified in the crystal structure of cytochrome oxidase from *Paracoccus denitrificans*, this is a phosphatidylcholine rather than a cardiolipin (*5*). However, the crystal structure of the photosynthetic reaction center from *Rhodobacter sphaeroides* shows the presence of a tightly bound cardiolipin molecule (*6*).

The headgroup of the lipid is also important in determining the phase preference of the lipid. While phospholipids such as the phosphatidylcholines adopt a lamellar, bilayer structure at normal temperatures, others, such as the phosphatidylethanolamines, can also adopt a curved, hexagonal H_{II} structure (*7*). Although this hexagonal structure is not compatible with formation of a bilayer membrane, lipids favoring the hexagonal H_{II} phase are often the major lipids in a membrane. For example, in the cell membrane of *Escherichia coli*, 72 wt % of the lipid is phosphatidylethanolamine, 24% is phosphatidylglycerol, and 4% is cardiolipin (*8*, *9*). The presence of relatively small amounts of a bilayer-favoring phospholipid (typically ∼10% or more) will force the phosphatidylethanolamine to adopt a bilayer structure, but the phosphatidylethanolamine is said to be in a state of frustration (*10*). It has been suggested that the presence of lipids in a state of frustration could be important for the proper function of intrinsic membrane proteins (*10*). For example, the presence of lipids that are able to form a hexagonal H_{II} phase seems to be required to produce the metarhodopsin II to metarhodopsin I ratio found for rhodopsin in the retinal rod membrane (*11*).

For the Ca^{2+}-ATPase of the sarcoplasmic reticulum, the presence of phosphatidylethanolamine is inhibitory (*12*). The Ca^{2+}-ATPase is relatively complex mechanistically, and changing phospholipid structure has been shown to affect many of the steps involved in the enzyme reaction (*12*). Dioleoylphosphatidylcholine [di(C18:1)PC][1] supports the highest rate of ATP hydrolysis for the Ca^{2+}-ATPase, although rather low levels of accumulation of Ca^{2+} were observed

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when the Ca^{2+}-ATPase was reconstituted into sealed vesicles of di(C18:1)PC alone (*12*, *13*). Much higher levels of accumulation of Ca^{2+} were observed when the Ca^{2+}-ATPase was reconstituted into bilayers containing 10% of an anionic phospholipid such as phosphatidylserine or cardiolipin, due to an effect of anionic phospholipids on the rate of slippage of the ATPase (*13*).

For studies of the effects of lipid−protein interactions on membrane protein function, there are obvious advantages in working with a kinetically simple system. In a previous paper, we have shown that diacylglycerol kinase (DGK) of *E. coli* can be reconstituted into lipid bilayers of defined composition and that the activity of DGK is sensitive to the chain length of the surrounding phospholipid (*14*). DGK is homotrimeric (*15*) and is predicted to contain just three transmembrane α-helices and two amphipathic helices (*16*). The mechanism of DGK is very simple. It binds its two substrates, ATP and diacylglycerol, in random order and carries out a direct phosphoryl transfer from ATP to diacylglycerol forming ADP and phosphatidic acid (*17*, *18*). Activity can be assayed very conveniently with dihexanoylglycerol (DHG) as the substrate; DHG is sufficiently hydrophobic that it is essentially all located within the lipid bilayer, and the chains are short enough that it does not phase separate in the bilayer or cause any detectable perturbation of the bilayer structure (*14*).

MATERIALS AND METHODS

Materials and General Procedures. Dimyristoleoylphosphatidylcholine [di(C14:1)PC], dipalmitoleoylphosphatidylcholine [di(C16:1)PC], dioleoylphosphatidylcholine [di(C18:1)PC], dioleoylphosphatidic acid [di(C18:1)PA], dioleoylphosphatidylethanolamine [di(C18:1)PE], dioleoylphosphatidylglycerol [di(C18:1)PG], dioleoylphosphatidylinositol [di(C18:1)PI], dioleoylphosphatidylserine [di(C18:1)PS], and cardiolipin were obtained from Avanti Polar Lipids. 1,2-Dihexanoylglycerol (DHG) was obtained from Sigma. Potassium cholate was purified by dissolving equimolar quantities of cholic acid and potassium hydroxide in methanol, followed by precipitation with excess diethyl ether.

A plasmid expressing His-tagged DGK was generously provided by J. Bowie (University of California, Los Angeles, CA). DGK was purified as described by Pilot et al. (*14*). DGK activity was measured using a coupled enzyme assay in which conversion of ATP to ADP is linked to the oxidation of NADH which was followed by the decrease in absorbance at 340 nm (*14*). The assay medium consisted of buffer [60 mM Pipes (pH 6.9)] containing phosphoenolpyruvate (2 mM), NADH (0.2 mM), ATP (5 mM), Mg^{2+} (20 mM), pyruvate kinase (18 units), and lactate dehydrogenase (22 units). The mixture was incubated at 25 °C for 10 min to ensure that any residual ADP in the ATP sample was consumed. The assay was initiated by addition of DGK (1.5 μg) to 1 mL of the assay medium. The oxidation of NADH was monitored by the decrease in absorbance at 340 nm.

DGK was reconstituted by mixing the lipid and DGK in cholate followed by dilution into buffer to form unsealed membrane fragments (*14*). Phospholipid (8 mol) and the required concentration of diacylglycerol (usually 2 μmol of DHG) were dried from a chloroform solution onto the walls of a thin glass vial. Buffer [400 μL of 60 mM Pipes (pH 6.9)] containing 28 mM cholate was added, and the sample was sonicated to clarity in a bath sonicator (Ultrawave). DGK (22 μg) was then added and the suspension left at room temperature for 15 min, followed by incubation on ice until the sample was use. Twenty microliters of the sample was then diluted into 1 mL of the assay buffer described above and DGK activity measured.

Table 1: Effects of Anionic Phospholipids on DGK Activity

system	activity (IU/mg)[a]
di(C18:1)PC	60.5
di(C18:1)PA	0.2
di(C18:1)PS	4.2
di(C18:1)PG	25.0
cardiolipin	4.3
80% di(C18:1)PE and 20% di(C18:1)PC	26.5
75% di(C18:1)PE, 20% di(C18:1)PG, and 5% cardiolipin	29.5
75% di(C18:1)PE, 15% di(C18:1)PG, and 10% cardiolipin	26.5

[a] Activities were measured at 5 mM ATP, 15 mM Mg^{2+}, and 20 mol % DHG at 25 °C.

RESULTS

Activity of DGK. The activity of DGK was followed by an enzyme-linked assay in which conversion of DHG to phosphatidic acid was linked to the oxidation of NADH, which was followed spectrophotometrically. In all cases, the decrease in the concentration of NADH was linear with time and the maximal change in NADH absorbance corresponded to the total conversion of the added DHG, as described by Pilot et al. (*14*).

Effects of Anionic Phospholipid. The activity of DGK reconstituted into bilayers of di(C18:1)PA, di(C18:1)PS, or cardiolipin is low, 10% or less of that in di(C18:1)PC (Table 1). In mixtures of di(C18:1)PC and di(C18:1)PS or cardiolipin, activity decreases fairly smoothly with increasing anionic phospholipid content, whereas in mixtures with di(C18:1)PA, activity is very low even at 20 mol % di(C18:1)PA (Figure 1).

DGK exhibits simple Michaelis−Menten kinetics with respect to both ATP and diacylglycerol (*14*, *17*, *18*). Reconstitution of DGK into lipid bilayers containing different proportions of DHG allows activity to be measured as a function of DHG concentration, expressed as the mole percentage in the lipid bilayer. Activities were measured at a MgATP concentration of 5 mM for DGK reconstituted into bilayers of di(C18:1)PC containing 20 mol % anionic phospholipid (Figure 2). The data fit well to a simple Michaelis−Menten scheme with the K_m and v_{max} values listed in Table 2. As shown, the presence of 20 mol % di(C18:1)PG had little effect on activity. The presence of 20 mol % di(C18:1)PS or di(C18:1)PI had little effect on v_{max} but caused a significant increase in K_m. In contrast, the presence of 20 mol % cardiolipin decreased activity through a decrease

[1] Abbreviations: DGK, diacylglycerol kinase; di(C14:0)PC, dimyristoylphosphatidylcholine; di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:0)PC, dipalmitoylphosphatidylcholine; di(C16:1)PC, dipalmitoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C18:1)PA, dioleoylphosphatidic acid; di(C18:1)PE, dioleoylphosphatidylethanolamine; di(C18:1)PG, dioleoylphosphatidylglycerol; di(C18:1)PI, dioleoylphosphatidylinositol; di(C18:1)PS, dioleoylphosphatidylserine; DM, *n*-decyl β-D-maltopyranoside; OG, octyl β-D-glucopyranoside; DHG, 1,2-dihexanoylglycerol.

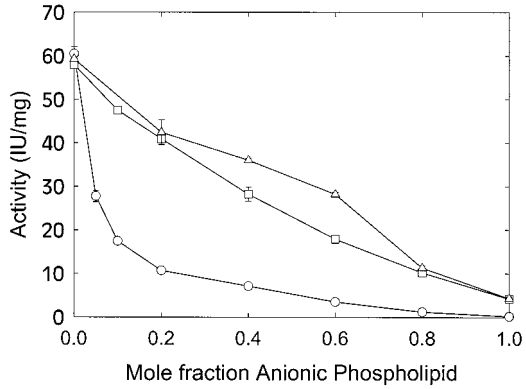

FIGURE 1: DGK activity in mixtures of di(C18:1)PC and anionic phospholipids. DGK was reconstituted with mixtures of di(C18:1)PC and di(C18:1)PA (○), di(C18:1)PS (□), and cardiolipin (△). Activities were measured at 25 °C with 5 mM ATP, 15 mM Mg^{2+}, and 20 mol % DHG.

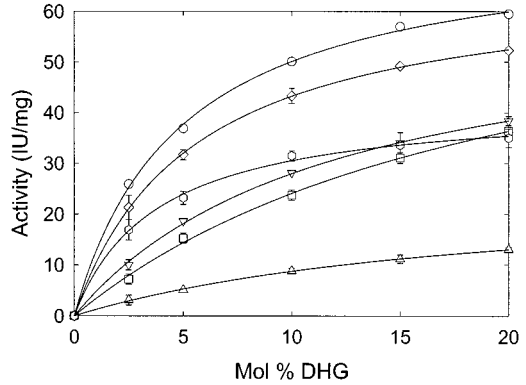

FIGURE 2: Effects of anionic phospholipids on DGK activity as a function of DHG concentration. DGK was reconstituted into mixtures of di(C18:1)PC containing 20 mol % anionic phospholipid at a constant phospholipid:DGK molar ratio of 6000:1 and the given mole percentage of DHG. Activities were measured at 25 °C with 15 mM Mg^{2+} and 5 mM ATP. DGK was reconstituted with di(C18:1)PC (○) and with mixtures of di(C18:1)PC containing 20 mol % of the following anionic phospholipids: (◇) di(C18:1)PG, (▽) di(C18:1)PI, (□) di(C18:1)PS, (△) di(C18:1)PA, and (⬡) cardiolipin. The solid lines show fits to the Michaelis−Menten equation with the values of K_m and v_{max} listed in Table 2.

Table 2: Effects of 20 mol % Anionic Phospholipids on v_{max} and K_m Values for DHG

anionic phospholipid[a]	v_{max} (IU/mg)	K_m (mol % DHG)
−	74.5 ± 1.2	4.9 ± 0.2
di(C18:1)PG	66.6 ± 0.5	5.4 ± 0.1
cardiolipin	42.1 ± 1.0	3.8 ± 0.3
di(C18:1)PS	74.4 ± 4.9	20.9 ± 2.3
di(C18:1)PI	61.2 ± 1.7	11.9 ± 0.7
di(C18:1)PA	25.3 ± 0.9	19.1 ± 1.2

[a] Bilayers contained di(C18:1)PC with 20 mol % of the anionic phospholipid. Activities were measured at 5 mM ATP and 15 mM Mg^{2+} at 25 °C.

in v_{max} with little effect on the K_m for DHG. Finally, the presence of di(C18:1)PA, the most inhibitory of the anionic phospholipids, resulted in both a decrease in v_{max} and an increase in K_m for DHG.

Activities as a function of ATP concentration at a fixed DHG concentration of 20 mol % also fitted well to Michaelis−Menten kinetics (Figure 3) with the K_m and v_{max} values listed in Table 3. Effects on K_m for ATP were rather small, with the K_m decreasing somewhat in cardiolipin. Mg^{2+}

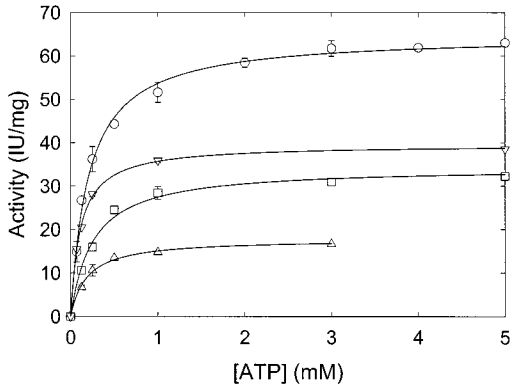

FIGURE 3: Effects of anionic phospholipids on DGK activity as a function of ATP concentration. DGK was reconstituted into mixtures of di(C18:1)PC containing 20 mol % anionic phospholipid at a constant phospholipid:DGK molar ratio of 6000:1 and 20 mol % DHG. Activities were measured at 25 °C with 15 mM Mg^{2+} and the given concentration of ATP. DGK was reconstituted with di(C18:1)PC (○) and with mixtures of di(C18:1)PC containing 20 mol % of the following anionic phospholipids: (□) di(C18:1)PS, (△) di(C18:1)PA, and (▽) cardiolipin. The solid lines show fits to the Michaelis−Menten equation with the values of K_m and v_{max} listed in Table 3.

Table 3: Effects of 20 mol % Anionic Phospholipids on v_{max} and K_m Values for ATP

anionic phospholipid[a]	v_{max} (IU/mg)	K_m (μM ATP)
−	64.8 ± 0.8	0.21 ± 0.01
cardiolipin	39.5 ± 0.4	0.11 ± 0.01
di(C18:1)PS	34.3 ± 0.9	0.25 ± 0.03
di(C18:1)PA	17.8 ± 0.4	0.18 ± 0.02

[a] Bilayers contained di(C18:1)PC with 20 mol % of the anionic phospholipid. Activities were measured at 20 mol % DHG and 15 mM Mg^{2+} at 25 °C.

is essential for the activity of DGK since the true substrate of DGK is MgATP; it has also been shown that, in detergent micelles, free Mg^{2+} is an activator of DGK activity with a K_a value of 3.4 mM (*18*). The activity of DGK in the reconstituted systems as a function of free Mg^{2+} concentration again fitted to simple Michaelis−Menten kinetics (Figure 4) with the K_a values listed in Table 4. The presence of 20 mol % cardiolipin had little effect on the K_a value, but the presence of 20 mol % di(C18:1)PS or di(C18:1)PA increased the K_a value by ∼3-fold.

Effect of Phospholipid Phase. The activity of DGK in mixtures of di(C18:1)PC and di(C18:1)PE decreases linearly with increasing di(C18:1)PE content up to 80 mol % di(C18:1)PE (Figure 5). Samples of DGK reconstituted into di(C18:1)PE as the only phospholipid were very turbid, probably due to the formation of nonbilayer phases; measurements of activity in these turbid samples were considered to be unreliable, but activities appear to be very low. Activities for DGK in mixtures of 80% phosphatidylethanolamine and 20% di(C18:1)PC were determined as a function of temperature (Figure 6A). At temperatures above ∼18 °C, di(C18:1)PE transforms from a bilayer to a hexagonal H_{II} phase (*7*). Activities of DGK in bilayers containing 80 mol % di(C18:1)PE are lower than those in a bilayer of di(C18:1)PC at the same temperature, the differences in activities increasing with increasing temperatures (Figure 6A). The temperature for the bilayer−hexagonal H_{II} phase transition for egg yolk phosphatidylethanolamine (28

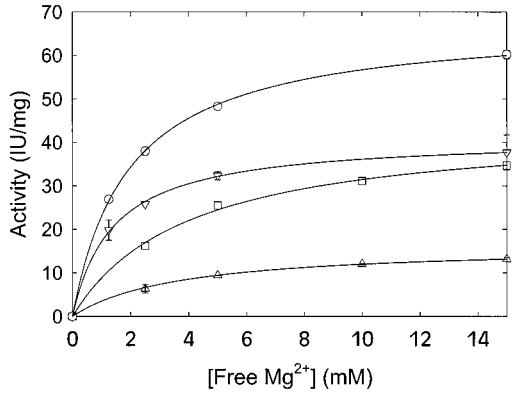

FIGURE 4: Effects of anionic phospholipids on DGK activity as a function of free Mg^{2+} concentration. DGK was reconstituted into mixtures of di(C18:1)PC containing 20 mol % anionic phospholipid at a constant phospholipid:DGK molar ratio of 6000:1 and 20 mol % DHG. Activities were measured at 25 °C with 5 mM ATP and the given concentrations of free Mg^{2+}. DGK was reconstituted with di(C18:1)PC (○) and with mixtures of di(C18:1)PC containing 20 mol % of the following anionic phospholipids: (□) di(C18:1)PS, (△) di(C18:1)PA, and (▽) cardiolipin. The solid lines show fits to the Michaelis−Menten equation with the values of K_a and v_{max} listed in Table 4.

Table 4: Effects of 20 mol % Anionic Phospholipids on v_{max} and K_a Values for Mg^{2+}

anionic phospholipid[a]	v_{max} (IU/mg)	K_a (mM Mg^{2+})
−	67.7 ± 0.6	1.9 ± 0.1
cardiolipin	41.2 ± 0.6	1.4 ± 0.1
di(C18:1)PS	44.0 ± 1.6	4.0 ± 0.4
di(C18:1)PA	16.3 ± 0.1	3. 7 ± 0.1

[a] Bilayers contained di(C18:1)PC with 20 mol % of the anionic phospholipid. Activities were measured at 20 mol % DHG and 5 mM ATP at 25 °C.

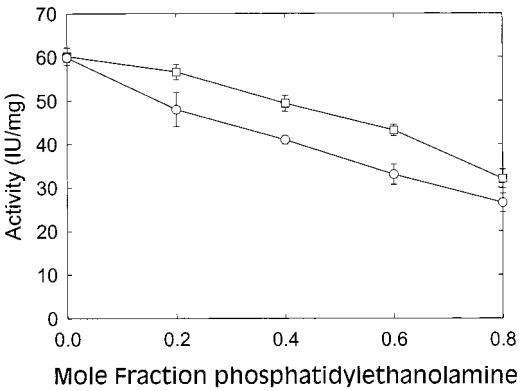

FIGURE 5: DGK activity in mixtures of di(C18:1)PC and phosphatidylethanolamines. DGK was reconstituted with mixtures of di(C18:1)PC and (○) di(C18:1)PE or (□) egg yolk phosphatidylethanolamine. Activities were measured at 25 °C with 5 mM ATP, 15 mM Mg^{2+}, and 20 mol % DHG.

°C) is higher than that for di(C18:1)PE (*19*). Activities of DGK in mixtures containing 80 mol % egg yolk phosphatidylethanolamine are equal to those in mixtures containing 80 mol % di(C18:1)PE up to ∼20 °C, beyond which activities become higher. These experiments show both that phosphatidylethanolamines support lower activities than the equivalent phosphatidylcholine and that the differences between the activities supported by phosphatidylethanolamines and phosphatidylcholines become more marked at

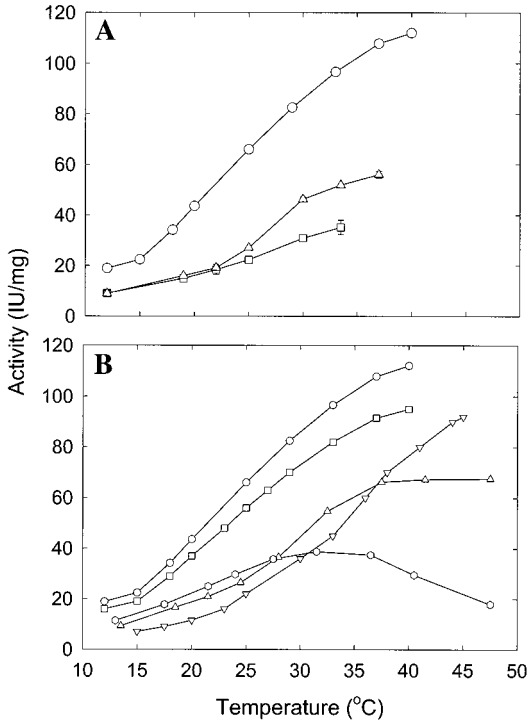

FIGURE 6: Effect of temperature on DGK activity in phosphatidylethanolamines and phosphatidylcholines. (A) DGK was reconstituted into di(C18:1)PC (○) or mixtures of 20 mol % di(C18:1)PC and 80 mol % di(C18:1)PE (□) or egg yolk phosphatidylethanolamine (△). (B) DGK was reconstituted into (○) di(C18:1)-PC, (▽) di(C16:0)PC, (□) di(C16:1)PC, (△) di(C14:0)PC, and (⬡) di(C14:1)PC. Activities were measured at 5 mM ATP, 15 mM Mg^{2+}, and 20 mol % DHG.

Table 5: Effects of Temperature on v_{max} and K_m Values for DHG[a]

phospholipid	temp (°C)	v_{max} (IU/mg)	K_m (mol % DHG)
di(C18: 1)PC	25	74.5 ± 1.2	4.9 ± 0.2
	30	108.6 ± 1.7	4.5 ± 0.2
80 mol % di(C18:1)PE	25	25.5 ± 0.6	2.4 ± 0.2
	30	50.8 ± 0.9	13.2 ± 0.5

[a] Activities were measured at 20 mol % DHG, 5 mM ATP, and 15 mM Mg^{2+} at 25 °C.

temperatures where the phosphatidylethanolamine alone would adopt a hexagonal H_{II} phase. Activities in mixtures containing 80 mol % di(C18:1)PE as a function of DHG concentration fitted well to simple Michaelis−Menten kinetics with the v_{max} and K_m values listed in Table 5.

Phosphatidylcholines with saturated fatty acyl chains undergo phase transitions between the fluid, liquid crystalline and solid, gel phases at temperatures that depend on fatty acyl-chain length. Phase transition temperatures for di(C14:0)PC and di(C16:0)PC are 24 and 42 °C, respectively (*7*). The activity for DGK in di(C16:0)PC is lower than that in di(C16:1)PC at all temperatures in the range of 15−45 °C (Figure 6B). The differences in activity decrease with increasing temperatures; at 20 °C, the activity in di(C16:1)-PC is 3 times that in di(C16:0)PC, whereas at 40 °C, the difference in activity is only 1.2-fold. Activities in di(C14:0)PC are lower than in di(C14:1)PC at temperatures below ∼30 °C, although the differences are rather small (Figure 6B). Above ∼35 °C, activities in di(C14:0)PC no longer increase with increasing temperatures, and in di(C14:1)PC, activities actually decrease with increasing temperatures

Table 6: Effects of Temperature on v_{max} and K_m Values for DHG in Saturated Phosphatidylcholines

		DHG[a]		ATP[b]	
phospholipid	temp (°C)	v_{max} IU/mg)	K_m (mol %)	v_{max} (IU/mg)	K_m (mM)
di(C14:0)PC	16	47.9 ± 4.2	27.9 ± 3.7	16.7 ± 0.4	0.24 ± 0.02
di(C16:0)PC	20	14.9 ± 0.3	3.1 ± 0.2		
	21			17.6 ± 0.4	0.38 ± 0.03

[a] Activities were measured at 5 mM ATP and 15 mM Mg^{2+}. [b] Activities were measured at 20 mol % DHG and 15 mM Mg^{2+}.

beyond ~35 °C (Figure 6B). Effects of low temperatures on v_{max} and K_m values for DHG and ATP in di(C14:0)PC and di(C16:0)PC are given in Table 6.

Effects of temperature were fully reversible. For example, when DGK was reconstituted in di(C14:1)PC and incubated at 42 °C, followed by addition to micelles of OG containing dioleoylglycerol as the substrate, the measured activity was the same as that for a sample of DGK that had not been reconstituted (data not shown).

DISCUSSION

Possible Effects of DHG on Phospholipid Phases. Long-chain diacylglycerols can have complex effects on the phase properties of phospholipids, including the formation of diacylglycerol−phospholipid complexes and conversion of phospholipids from a bilayer to a hexagonal H_{II} phase (*20*−*22*). We have therefore chosen to use the short-chain DHG as a substrate because short-chain diacylglycerols such as DHG are miscible with phospholipids in the liquid crystalline phase and have no effect on the properties of the lipid bilayer (*23*, *24*). Further, studies of DGK in detergent micelles have shown that long-chain diacylglycerols can act both as a substrate and as a lipid activator of the protein, complicating analysis of the effects of phospholipids (*25*). In the presence of activating phospholipids, short-chain diacylglycerols which are not themselves activators of DGK are still substrates of the enzyme, with reduced K_m values but the same v_{max} values (*26*).

Although the effects of short-chain diacylglycerols on the phase properties of lipids such as phosphatidylethanolamine that favor the hexagonal H_{II} phase do not appear to have been reported in the literature, the design of the experiments reported here suggests that all systems are likely to remain in the bilayer phase. We observed that mixtures of di(C18:1)PE containing 20 mol % DHG gave very cloudy samples, typical of the formation of a hexagonal H_{II} phase. However, mixtures with a 1:4 di(C18:1)PC:di(C18:1)PE molar ratio containing 20 mol % DHG did not form cloudy suspensions in water, consistent with the well-established ability of phosphatidylcholine to favor a bilayer structure (*27*). Further, the presence of integral membrane proteins such as glycophorin A (*28*), cytochrome oxidase (*29*), and the light-harvesting complex of photosystem 2 (*30*) have all been shown to stabilize lipids favoring the hexagonal H_{II} phase in a bilayer structure. In the cytochrome oxidase/cardiolipin system, it was found that under conditions where some hexagonal H_{II} phase was formed, cytochrome oxidase was excluded from the hexagonal H_{II} phase and was located in vesicular structures in which the lipid was organized as a bilayer (*29*).

In all the experiments reported here, linear kinetics were observed with all the DHG substrate being consumed, suggesting the formation of homogeneous samples. A bulk phase separation into hexagonal H_{II} and bilayer phases with no exchange of DHG between the phases would result in only partial consumption of the DHG, which was not observed. Exchange of DHG between bilayers of phosphatidylcholine is slow (*14*); a bulk phase separation with slow exchange of DHG between the phases is also unlikely, since this would result in biphasic kinetics, which were not observed. It was also found, as described, that plots of DGK activity against DHG concentration all fitted to simple Michaelis−Menten kinetics. If addition of DHG resulted in conversion of bilayers into a hexagonal H_{II} phase lipid and if this affected the activity of DGK, breaks in the Michaelis−Menten plots would be observed at the concentration of DHG at which the hexagonal H_{II} phase first formed. The fact that such breaks were not seen suggests that no phase changes of importance for activity were produced by addition of DHG. Thus, we assume that the lipid environment of DGK in these experiments remains as a bilayer and that if any hexagonal H_{II} phase formed then exchange of DHG between the hexagonal H_{II} and bilayer phases is sufficiently fast not to affect the kinetics.

Anionic Phospholipids. DGK carries out a two-phase reaction between water-soluble ATP and a water-insoluble diacylglycerol. The insoluble nature of the diacylglycerol substrate means that DGK has to be assayed in the presence of detergent micelles or lipid bilayers in which DGK and the diacylglycerol can mix. DGK in micelles of the detergent octyl glucoside showed very low activity with dioleoylglycerol as a substrate in the absence of added phospholipid (*18*). Addition of cardiolipin, di(C18:1)PC, or di(C18:1)PG led to a large increase in activity, with an amount of cardiolipin smaller than the amounts of the other lipids being required to increase activity. It was therefore suggested that cardiolipin could bind to a small number of sites on DGK with high affinity, whereas interaction with the other lipids was less specific (*18*). Di(C18:1)PS was somewhat less effective at activating DGK than the other phospholipids, and the effect of di(C18:1)PA on activity was much smaller (*18*). Bohnenberger and Sandermann (*31*) obtained somewhat different results by assaying DGK activity in micelles of Triton X-100 with dipalmitoylglycerol as the substrate. Again, cardiolipin was found to be the most efficient phospholipid at activating DGK, but activation at low concentrations of cardiolipin was followed by inhibition at higher concentrations; on the other hand, little inhibition was observed at high concentrations of phosphatidylglycerol or phosphatidylcholine. The maximal activity measured in the presence of phosphatidylethanolamine was approximately half of that observed in cardiolipin (*31*).

Although, as already described, anionic phospholipids activate DGK in detergent micelles, in lipid bilayers cardiolipin, di(C18:1)PA, and di(C18:1)PS support very low activities compared to that supported by di(C18:1)PC (Figure 1 and Table 1). Binding of Mg^{2+} to di(C18:1)PA results in the formation of a nonlamellar structure in which the lipid fatty acyl chains are highly ordered (*32*). In contrast, addition of Mg^{2+} to di(C18:1)PS results in no change in chain packing (*33*, *34*). The lower activities observed for DGK in di(C18:1)PA than in di(C18:1)PS (Table 1) could follow from

formation of ordered structures of di(C18:1)PA in the presence of high concentrations of Mg^{2+}. However, Mg^{2+} has little effect on anionic phospholipids when they are present in a mixture with a zwitterionic phospholipid (*33*, *35*), and so the inhibitory effects observed for mixtures of di(C18:1)PC containing 20 mol % anionic phospholipid are unlikely to be Mg^{2+}-dependent (Figure 1). Indeed, measurement of DGK activity as a function of Mg^{2+} concentration in these systems shows activity increasing with increasing Mg^{2+} concentration (Figure 4 and Table 4), which can be attributed to the activator role for Mg^{2+} previously reported in detergent micelles (*18*).

The reasons for the low activities observed in mixtures containing anionic phospholipid are different for the different phospholipids. For mixtures containing di(C18:1)PS and di(C18:1)PI, low activities follow largely from an increase in K_m for DHG with little effect on v_{max} (Table 2). The low activity in the mixture containing cardiolipin follows largely from a decrease in v_{max} with a slight decrease in the K_m for DHG. The low activity in the mixture containing di(C18:1)PA follows from a decrease in both the v_{max} and K_m for DHG. Finally, the presence of di(C18:1)PG at 20 mol % has little effect on activity; this is potentially important since phosphatidylglycerol is the major anionic phospholipid in the *E. coli* cell membrane.

Since the product of the DGK reaction is phosphatidic acid, it is possible that the large effect of di(C18:1)PA on the K_m for DHG follows from binding of di(C18:1)PA at the active site in competition with DHG. The increase in K_m for DHG observed with di(C18:1)PS and di(C18:1)PI would then suggest that these anionic phospholipids can also bind to the active site. The lack of an effect of cardiolipin on the K_m for DHG would suggest that cardiolipin is excluded from the active site, presumably because it is much bulkier than the other phospholipids, containing, as it does, four fatty acyl chains.

Only cardiolipin and phosphatidic acid have a significant effect on v_{max} (Table 2). Since DGK catalyzes the direct transfer of the γ-phosphate of ATP to the OH group of the bound diacylglycerol, it is likely that the two substrates are bound closely together at the subunit−subunit interface in the DGK trimer where the active site is located (*15*). It is possible therefore that binding of di(C18:1)PA or cardiolipin to DGK results in a conformation change on the enzyme, changing the relative locations of the ATP and diacylglycerol binding sites at the interface, reducing the rate of phosphoryl transfer. Comparing the results obtained here for the effects of cardiolipin on activity in bilayers of phosphatidylcholine with those of Walsh and Bell (*18*) for activity in micelles of octyl glucoside is difficult, but the maximum activity measured by Walsh and Bell (*18*) in the presence of cardiolipin was lower than that measured in the presence of other anionic lipids, suggesting that some inhibition of activity by cardiolipin in octyl glucoside might have occurred, as well as the general phospholipid-activating effect.

Effects of Phosphatidylethanolamines. All biological membranes contain lipids which, when isolated from the membrane, prefer to adopt a curved hexagonal H_{II} structure rather than a planar bilayer structure. In mixtures with lipids preferring a bilayer phase, these non-bilayer-preferring lipids are forced to adopt a bilayer structure and therefore exist in a state that has been termed one of curvature frustration; it has been suggested that the presence of bilayer frustration is important for the proper function of membrane proteins (*10*).

In mixtures of di(C18:1)PC and di(C18:1)PE at 25 °C, DGK activity decreases almost linearly with increasing di(C18:1)PE content up to 80 mol % di(C18:1)PE (Figure 5). Egg yolk phosphatidylethanolamine (predominantly 1-palmitoyl-2-oleoylphosphatidylethanolamine), which has a higher liquid crystalline to hexagonal H_{II} phase transition temperature, has a slightly less inhibitory effect on DGK activity than di(C18:1)PE (Figure 5). Effects of phosphatidylethanolamines are more unfavorable at temperatures that favor formation of the hexagonal H_{II} phase than at temperatures that favor the bilayer phase. Thus, activities in mixtures containing di(C18:1)PE and egg yolk phosphatidylethanolamine are the same at temperatures up to ∼25 °C, but then activities become lower in mixtures containing di(C18:1)PE than in mixtures containing egg yolk phosphatidylethanolamine (Figure 6A). At 25 °C, a temperature close to the bilayer to hexagonal H_{II} phase transition for di(C18:1)PE, low activities follow from a low v_{max}, the K_m value for DHG being slightly lower than in di(C18:1)PC (Table 5). However, at higher temperatures, the low activity follows from both a low v_{max} and a high K_m value; for phosphatidylcholines, temperature has no significant effect on the K_m for DHG (Table 5).

Effects of a Gel Phase Phospholipid. Many membrane proteins exhibit low activity when the surrounding lipid is in the gel phase. For example, the Ca^{2+}-ATPase of the sarcoplasmic reticulum reconstituted into bilayers of di(C14:0)PC is inactive at <24 °C, the temperature of the gel to liquid crystalline phase transition for this lipid (*36*). Similarly, the Ca^{2+}-ATPase is inactive in di(C16:0)PC at low temperatures, but in this case, activity appears above ∼32 °C even though the phase transition temperature for di(C16:0)PC is 42 °C (*36*). The glucose transporter from red blood cells also shows no activity in gel phase di(C14:0)PC, although there is activity in di(C18:0)PC in the gel phase (*37*).

The activity of DGK in bilayers of di(C16:0)PC at temperatures below ∼45 °C is lower than that in di(C16:1)PC, suggesting that the gel phase lipid supports low activity (Figure 6). The decrease in activity with decreasing temperatures in di(C16:0)PC is gradual with no sharp change at 42 °C, suggesting that the phase transition for the lipid around DGK is broad, as has been suggested previously for the Ca^{2+}-ATPase and other membrane proteins (*38*). At low temperatures in di(C16:0)PC, K_m values for DHG and ATP are close to normal, the major effect of temperature being a large decrease in v_{max} (Table 6). Effects of gel phase di(C14:0)PC are more complex (Figure 6). Activities in both di(C14:1)PC and di(C14:0)PC at <24 °C are low, and the activity in di(C14:0)PC is only slightly lower than that in di(C14:1)PC. However, the reasons for the low activities in di(C14:0)PC and di(C14:1)PC are different. It was shown previously that the low activity in di(C14:1)PC followed from a high K_m value for DHG [64.5 mol % in di(C14:1)PC compared to 4.9 mol % in di(C18:1)PC)] with a value for v_{max} similar to that in di(C18:1)PC. In contrast, in gel phase di(C14:0)PC, the K_m value for DHG is only 27.9 mol % but the value for v_{max} is reduced (Table 6). The smaller effect of gel phase di(C14:0)PC than of liquid crystalline phase di(C14:1)PC on the K_m for DHG is likely to follow from effects of lipid

bilayer thickness. The thickness of a bilayer in the gel phase is ~30% greater than that for a bilayer in the liquid crystalline phase (*39*) so that the thickness of a bilayer of di(C14:0)PC in the gel phase will be comparable to that of a bilayer of di(C18:1)PC in the liquid crystalline phase; this increase in bilayer thickness could explain the decrease in the K_m value from di(C14:1)PC to di(C14:0)PC (Table 6). The low value for v_{max} in a gel phase lipid could reflect a misalignment of ATP and diacylglycerol at the active site of DGK, as suggested above for the effects of cardiolipin and phosphatidic acid, or it could reflect a high activation energy for a kinetically important conformation change on DGK in the rigid environment provided by a gel phase lipid bilayer.

Activity in the Native Membrane. In the native *E. coli* cytoplasmic membrane, ~72 wt % of the lipid is phosphatidylethanolamine, 24% is phosphatidylglycerol, and 4% is cardiolipin (*8*, *9*). Activities for DGK in bilayers of this composition are about half that observed in a bilayer of di(C18:1)PC when measured with 20 mol % DHG and 5 mM ATP (Table 1). The activities in mixtures containing high proportions of di(C18:1)PE are the same whether the other lipid(s) is di(C18:1)PC or a mixture of di(C18:1)PG and cardiolipin, showing that the dominant effect is that of di(C18:1)PE. Low activities in the presence of di(C18:1)PE at 30 °C follow from both a high K_m value for DHG and a low v_{max} value (Table 5). The importance of the effect on K_m may be lessened when the substrate for DGK is the natural, longer-chain diacylglycerol since this will have a K_m value lower than that for DHG. Nevertheless, the value for v_{max} is lower in di(C18:1)PE than in di(C18:1)PC, and v_{max} values are expected to be independent of the chain length of the diacylglycerol (*26*). Comparison with the native membrane is made difficult by the asymmetric lipid distribution between the two leaflets of the lipid bilayer. Either this has a significant effect on DGK activity, or the lipid environment of DGK in the *E. coli* membrane is less than optimal as far as the rate of reaction is concerned.

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