Correlation between Bilayer Lipid Dynamics and Activity of the Diglucosyldiacylglycerol Synthase from *Acholeplasma laidlawii* Membranes[†]

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Received February 7, 1996; Revised Manuscript Received May 17, 1996[⊗]

ABSTRACT: In the single membrane of *Acholeplasma laidlawii* a specific glucosyltransferase synthesize the major, lamellar-forming lipid diglucosyldiacylglycerol (DGlcDAG) from the major, nonlamellar-prone monoglucosyldiacylglycerol (MGlcDAG). This is crucial for the maintenance of phase equilibria close to a bilayer−nonbilayer transition and a nearly constant spontaneous curvature in the membrane lipid bilayer. Acyl chain order is also affected, but not kept constant. Phosphatidylglycerol (PG) is an essential activator, needed in substantial amounts by the DGlcDAG synthase, and likely to affect bilayer properties. A potential connection was investigated between the (i) lateral diffusion, (ii) domain formation of the PG activator, and (iii) bilayer chain ordering (i.e., the hydrocarbon free volume), revealed in unilamellar liposomes by lipid probes containing one or two (fluorescent) pyrene acyl chains, *and* (iv) activity of the DGlcDAG synthase. Different activator, nonbilayer perturbant, and bilayer matrix conditions were employed. *Diffusion* of PG was substantially slower in a DGlcDAG compared to a phosphatidylcholine (PC) matrix with 18:1c chains but increased with the PG content in both. No obvious correlation between diffusion and enzyme activity, and no local concentration of PG as a function of chain ordering or curvature, was detected. However, an enrichment of PG activator into *domains* could be induced by a chain length mismatch between 18:1c-PG and 14:1c-PC (but not 22:1c-PC), even at small PG fractions. Patching was sufficient to stimulate enzyme activity 4-fold in relation to the activities normally valid at low PG concentrations. *Chain order* was substantially lower (i.e., free volumes larger) in bilayers of DGlcDAG than in bilayers of PC and increased in an additive fashion in both by the content of especially the nonbilayer-prone 1,3-18:1c-DAG but also by PG. At physiological concentrations of PG in DGlcDAG bilayers (≈20%) a good correlation was evident between increased DAG content and chain ordering and strongly enhanced enzyme activities, with maxima close to a bilayer−nonbilayer transition. It is concluded that regulation of packing conditions in *A. laidlawii* membranes by the DGlcDAG synthase seems to be governed not by the absolute extent of chain order but more by the spontaneous curvature within a certain range of conditions. Domain formation of the essential PG activator due to bilayer conditions is a second mechanism, potentially overriding the curvature effects.

In biological membranes the composition of hydrocarbon chains and polar headgroups in the bilayer lipids are carefully regulated at the enzyme level, often yielding different lipid composition in the various intracellular membranes (Hjelmstad & Bell, 1991). This is likely to affect the optimal function of many membrane proteins. However, the nature of bilayer properties maintained by the lipid-synthesizing enzymes, and the mechanisms for their sensing, are less understood. In the single membrane of the small prokaryote *Acholeplasma laidlawii* an extensive metabolic regulation in the amounts of the major phospholipids and glucolipids occurs under a variety of conditions (Rilfors et al., 1993). This serves to maintain (i) a constant surface charge density (Christiansson et al., 1985), (ii) phase equilibria close to a potential lamellar (L_α)[1] to nonlamellar transition (Lindblom et al., 1986), and (iii) a nearly constant radius of spontaneous curvature (Österberg et al., 1995), respectively, as analyzed the for the liquid-crystalline bilayer lipid mixture *in vitro*. Similar packing conditions for lipid bilayer molecules are probably also crucial in other, unrelated bacteria such as *Escherichia coli* (Rietveld et al., 1993), some clostridia (Goldfine, 1993), and *Bacillus megaterium* (Rilfors et al., 1994). These latter species have different polar lipids and partly different lipid hydrocarbon chains from *A. laidlawii*,

[†] This work was supported by the Swedish Natural Science Research Council, the Knut and Alice Wallenberg Foundation, the Nordic Research Education Academy (NorFA), the Medical Research Council of the Academy of Finland, Biocenter Helsinki, the Sigrid Juselius Foundation, and the Research and Science Foundation of Farmos.

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[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: Lipid acyl chains: 14:1c, tetradecenoyl (9-*cis*); 16:1c, hexadecenoyl (9-*cis*); 18:1c, octadecenoyl (9-*cis*) (or oleoyl); 20:1c, eicosenoyl (11-*cis*); 22:1c, docosenoyl (13-*cis*); DAG, 1,2-diacylglycerol; DGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1 → 2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; DOG, 1,3-dioleoylglycerol; Glc, glucose; L_α, lamellar liquid-crystalline phase; MGlcDAG, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PPDPC, 1-palmitoyl-2-[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine; PPDPG, 1-palmitoyl-2-[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac′-glycerol; bisPDPC, 1,2-bis[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine; bisPDPG, 1,2-bis[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac′-glycerol; TLC, thin-layer chromatography.

S0006-2960(96)00287-5 CCC: $12.00

and regulation occurs mainly at the acyl chain level, indicating the importance of the packing principles and not the exact chemical structures of the participating molecules (cf. Wieslander, 1996).

Key lipids in *A. laidlawii* for regulation of the packing properties are monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), forming mainly nonlamellar and L_α phases at physiological conditions, respectively (Lindblom et al., 1986; Mannock et al., 1990; Foht et al., 1995; Wieslander et al., 1995). These are made from 1,2-DAG by two consecutive glucosyl-transfer reactions (Smith, 1969; Dahlqvist et al., 1992). The DAG is made from phosphatidic acid (PA), which is also the precursor for the separate pathway to the major, L_α-forming phosphatidylglycerol (PG). DAG, a potent nonbilayer lipid, may accumulate in membranes with saturated acyl chains of short and medium length (Wieslander et al., 1995). The metabolic flow from the PA precursor, by way of DAG sequentially to MGlcDAG and DGlcDAG, and then to two (usually minor) phosphoglucolipids, depends on several factors. *In vivo* higher growth temperatures, or unsaturated and longer lipid acyl chains in the bilayer, yield more DGlcDAG and less MGlcDAG (Wieslander et al., 1995), as do the presence in the membrane of various foreign nonbilayer-promoting molecules (Wieslander et al., 1986). This is in accordance with the rules for the molecular packing and phase equilibria of amphiphiles in general (Israelachvili, 1992; Gruner, 1992).

In vitro the MGlcDAG synthase is critically dependent upon the presence of a substantial fraction of anionic phospholipids like PG for activity (Karlsson et al., 1994; Dahlqvist et al., 1995). This seems to constitute a rate-keeping connection between the PG and glucolipid pathways *in vivo*, which yields the constant surface charge density of the lipid bilayer (Christiansson et al., 1985; Wieslander et al., 1995). The DGlcDAG synthase is dependent upon PG specifically, in an essential and cooperative manner *in vitro* (Karlsson et al., 1994). Demands for PG varies with the acyl chain types of the PG activator and the type of bilayer matrix and is strongly modulated by changes in the spontaneous curvature due to different nonbilayer-prone molecules, in a dose-dependent manner (Karlsson et al., 1994; Dahlqvist et al., 1995). The changes in rates of synthesis of DGlcDAG by the latter molecules are in agreement with and sufficient for the metabolic changes observed *in vivo*. The large amounts of PG needed for the activation of the DGlcDAG synthase ($\geq$20 mol %; Dahlqvist et al., 1995) indicate that a certain change of bilayer surface properties brought by PG, locally or totally, may be an additional factor for regulation of enzyme activity; a single, high-affinity PG/synthase interaction is less likely (cf. Newton, 1993). *In vivo* a correlation between amounts of PG (5−27 mol %) and DGlcDAG (5−50 mol %) was observed in cells with membrane acyl chains of different length and extent of unsaturation (Wieslander et al., 1995). However, PG never experience rapid metabolic changes in amounts as can DGlcDAG (and MGlcDAG). Hence, other factors are probably also involved in regulation of the DGlcDAG synthase activity, like the chain ordering, domain formation, or the spontaneous curvature in the bilayer.

We report here, from studies with pyrene chain-labeled lipids in well-defined liposomal systems close to *A. laidlawii* cell size, changes in lipid dynamics, i.e., variation in lateral diffusion, microdomain formation, and chain−chain interactions in PG and different endogenous and synthetic bilayer matrix lipids under a variety of conditions. A close correlation was observed between (i) the DGlcDAG synthase activity, reduction in bilayer free volume, including increased chain ordering (Xiang, 1993), and phase equilibria in certain di-18:1c lipid bilayer systems, respectively, and (ii) synthase activity and the local patching of the di-18:1c-PG activator in thin but not in thick bilayers. This is in accordance with observations *in vivo* and shows that the properties and behavior of the PG activator are of fundamental importance.

MATERIALS AND METHODS

Organism and Growth Conditions. *A. laidlawii* strain A-EF22 was cultivated in an oleic acid (18:1c)-supplemented medium and harvested, and the protein content of cells and membranes was determined, as described by Dahlqvist et al. (1995).

Matrix and Substrate Lipids. The MGlcDAG and DGlcDAG lipids used here were prepared from *A. laidlawii* grown in the presence of oleic acid, which gives a lipid preparation with more than 90% (mol/mol) 18:1c acyl chains (Dahlqvist et al., 1995). 1,2-18:1c-DAG and 1,3-18:1c-DAG were purchased from Larodan (Malmö, Sweden). Phosphatidylglycerol (sodium salt), di-18:1c-phosphatidylethanolamine (di-18:1c-PE), and all of the various phosphatidylcholines (PCs) were purchased from Avanti Polar Lipids (Alabaster, AL).

Pyrene-Labeled Lipids. 1-Palmitoyl-2-[10-(pyren-1-yl)]-decanoyl-*sn*-glycero-3-phosphocholine (PPDPC), 1-palmitoyl-2-[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac′-glycerol (PPDPG), 1,2-bis[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphocholine (bisPDPC) and 1,2-bis[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phospho-rac′-glycerol (bisPDPG) were purchased from K & V Bioware (Espoo, Finland).

Preparation of Liposomes. Lipid mixtures of desired composition, solubilized in chloroform, 1,1,1-trichlorethylene, or chloroform/methanol 2:1 (v/v), were dried under a stream of nitrogen (gas) and then under reduced pressure for more than 2 h. The dry lipids were hydrated in 250 μL of buffer (1 mM lipid concentration) containing 20 mM HEPES, 100 mM sodium maleate, and 20 mM $MgCl_2$ (when present), pH 8, by repeated agitation during 45 min. A glass bead was added to the tubes in order to make the agitation more efficient. Large unilamellar vesicles (100 nm) (LUVs) were made by 19 successive extrusions of the lipid dispersions through double 100 nm pore size polycarbonate filters (Nuclepore, Pleasanton, CA) with a LiposoFast small volume homogenizer (Avestin, Ottawa, ON). At high molar fractions of nonbilayer-prone lipids present (e.g., >25 mol % DOG or >80 mol % di-18:1c-PE), there were severe difficulties in hydrating the lipid mixtures, which came off the tube walls as turbid aggregates. Sonication of such mixtures only caused further aggregation or had no visible effect; some of these aggregated lipids also got stuck in the filters during extrusion. Consequently, such lipid aggregates were not included in the analyses (below).

Fluorescence Measurements. The liposome preparations (with the indicated probes) were diluted 40 times with 20 mM HEPES, 100 mM sodium maleate, and 20 mM $MgCl_2$ (if present), pH 8, to $\sim$25 μM final lipid concentration, in a magnetically stirred 1 × 1 cm four-window quartz cuvette. After temperature equilibration in the thermostated holder

at 28 °C, the samples were excitated at 344 nm and emission spectra were collected between 360 and 600 nm. Slits of 5 nm were used for both the excitation and emission beams. Measurements using phospholipids with pyrene-labeled acyl chains to probe, e.g., lateral distribution and mobility of lipids, are widely employed and accepted [reviewed in Kinnunen et al., (1993)]. The excited monomeric pyrene is emitting maximally at ~394 nm (I_m) but if forming an excimer complex with a ground state pyrene the complex will reach the relaxed state emitting maximally at ~480 nm (I_e) (Förster, 1969). The excimer formation using lipid analogues with one pyrene-acyl chain is an *intermolecular* event, depending on the collision rate of these molecules, which means that the I_e/I_m ratio mirrors their lateral mobility and concentration (Galla & Hartmann, 1980). Excimer formation using lipid analogues with two pyrene-acyl chain is, on the other hand, an *intramolecular* event at low probe bilayer concentrations (e.g., 0.1% mol/mol) (Sunamoto et al., 1980). The rate of eximer formation increases with increasing frequency of chain splaying motions and also upon reduction in their amplitude. Therefore, at constant temperature the inverse of the I_e/I_m ratio is here a measurement of the free volume (Lehtonen & Kinnunen, 1994) and thus also chain order in the hydrophobic core of the lipid bilayer (Xiang, 1993).

Assay for DGlcDAG Synthesis. The enzymatic activity of the DGlcDAG synthase was analyzed in reconstituted (liposome) bilayers. Protein mixtures from membranes where 96% of the native lipids had been removed by solvent extraction (Karlsson et al., 1994; Dahlqvist et al., 1995) were used. Exogenous lipid mixtures of selected compositions, dissolved in benzene, were added to 0.5 mg aliquots of these extracted preparations, and the benzene was evaporated under a stream of nitrogen (Karlsson et al., 1994). After incubation of the dry mixtures at 4−8 °C for 30 min, 80 μL of 0.15 M Tris-maleate (pH 8) was added and violently agitated, followed by 10 μL of 0.2 M $MgCl_2$. Finally, 10 μL of 10 mM (1.0 Ci/mol) UDP[^{14}C]glucose was added to start the enzyme reactions (Dahlqvist et al., 1992), and the mixtures were incubated for 10 min at 28 °C. The reactions were stopped by the addition of 375 μL of methanol/chloroform 2:1 (v/v), and the synthesized DGlcDAG was extracted and then separated by thin-layer chromatography (TLC) as described by Karlsson et al. (1994). The DGlcDAG on the TLC plates was visualized and quantified by electronic autoradiography (Packard InstantImager). The purified MGlcDAG substrate (above) and the enzymatically synthesized DGlcDAG product migrated as single spots with unique R_f values corresponding to synthetic or structurally determined MGlcDAG and DGlcDAG in two different TLC solvent systems (Mannock et al., 1990; Hauksson et al., 1995). The synthesis rate for DGlcDAG was expressed as nanomole of lipid synthesized per milligram of protein per hour, as calculated from the amount of radioactive glucose incorporated into the lipid.

RESULTS AND DISCUSSION

Factors Important for DGlcDAG Synthesis. Several factors are expected to influence the activity of the DGlcDAG synthase by affecting membrane bilayer properties. *(i) Type of bilayer matrix lipid.* DGlcDAG has a substantially larger polar head, but less headgroup hydration, than the common phosphatidylcholine (PC). However, both form lamellar phases. Furthermore, glycolipid polar heads are likely to interact by extensive intermolecular hydrogen bonding, as supported by their chemical and crystal structures; this is not the case for PC heads (Pascher et al., 1992). *(ii) Presence of activator lipid.* Phosphatidylglycerol is an essential activator of the enzyme in bilayers; no other anionic phospholipid works, not even those containing phosphoglycerol headgroup moities. PG is also important for the surface charge density in *A. laidlawii* (Christiansson et al., 1985) and can participate in hydrogen bonding (Pascher et al., 1992). *(iii) Lateral diffusion and domain formation.* Bacterial cells (Chang et al., 1986; Coughlin et al., 1983), and *A laidlawii* membranes (Kahane et al., 1973) contain substantial amounts of Mg^{2+}. It is an essential cofactor for the DGlcDAG synthase activity (Dahlqvist et al., 1992) and can affect the physical properties of PG (Findlay & Barton, 1978) and DGlcDAG (Wieslander et al., 1978). The interactions of PG and the related glycolipid dimannosyl-DAG *in vitro* was affected by Mg^{2+} (Lakhdar-Ghazal & Tocanne, 1981). These two lipids revealed a heterogeneous lateral distribution in a bacterial membrane (de Bony et al., 1989), which may change during the cell cycle (Welby et al., 1996), indicating a potential for domain formation. *(iv) Chain order and spontaneous curvature.* The synthesis of DGlcDAG *in vivo* is stimulated at conditions of increased chain length and unsaturation and by the presence of nonbilayer lipid additives. Increased length increases chain order and curvature, whereas increased unsaturation decreases order but increases curvature, respectively. Nonbilayer-prone lipids usually force lipid chains to an increased order due to their orientation in the bilayer and their smaller interfacial areas. These conditions are intimately connected to an increased (negative) spontaneous curvature of the bilayers; the ultimate source of this stress is a mismatch between the lipid polar and nonpolar cross-sectional molecular areas (Gruner, 1992, 1994).

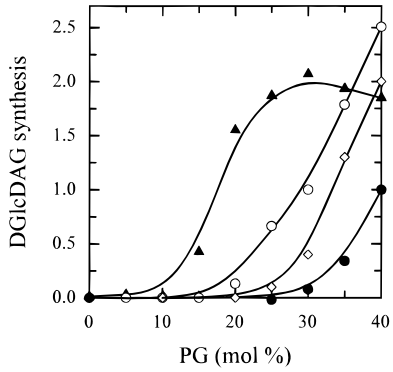

FIGURE 1: Synthesis of DGlcDAG in different bilayer matrixes. The reconstituted membranes contained 10 mol % di-18:1c-MGlcDAG substrate and 0−45% di-18:1c-PG in a matrix of di-18:1c-PC (●), di-18:1c-PC plus 15 mol % di-18:1c-PE (◇), di-18:1c-DGlcDAG (○), or di-18:1c-DGlcDAG plus 15 mol % 1,3-DOG (▲), respectively. DGlcDAG synthesis was started by addition of UDP-Glc (see Materials and Methods). Mean values from three experiments, normalized to the value for 30% PG in DGlcDAG.

The fraction of PG present was essential for the activity of the DGlcDAG synthase in reconstituted lipid bilayers (Figure 1). The type of matrix lipid also matters: the native di-18:1c-DGlcDAG was substantially more efficient than the foreign di-18:1c-PC. Relatively small amounts of 1,3-18:1c-DAG (1,3-DOG), a potent nonbilayer lipid (van Gorkom et al., 1992) structurally similar to the 1,2-DAG endogenous

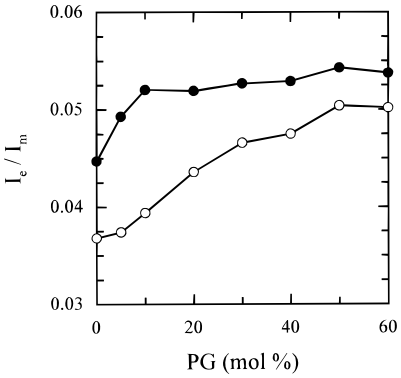

FIGURE 2: Lateral diffusion of PG. Changes in I_e/I_m was monitored from PPDPG as a function of increasing di-18:1c-PG concentrations in a di-18:1c-DGlcDAG (○) or di-18:1c-PC (●) matrix. The probe concentration was 1 mol %; liposome preparation and fluorescence measurements were as described in Materials and Methods. Absence of Mg^{2+} (normally 20 mM) increased the I_e/I_m from PPDPG in di-18:1c-PC 10−15%, and the presence of 10 mol % 1,3-DOG in DGlcDAG caused an increase of approximately 10%, at the indicated PG fractions (data not shown). I_e/I_m from PPDPC in di-18:1c-PC was little affected by Mg^{2+} or the presence of PG.

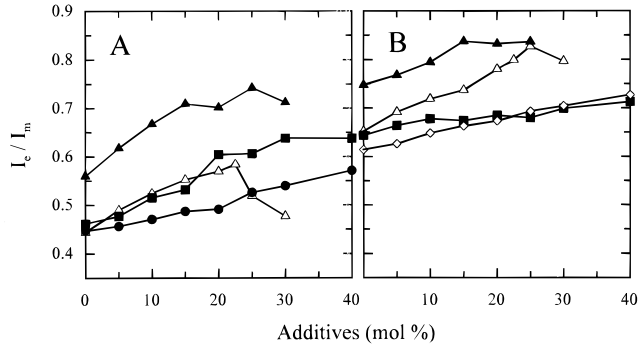

FIGURE 3: Chain ordering by PG, 1,3-DOG and different matrixes. Changes in I_e/I_m from bisPDPC and bisPDPG was monitored as a function of increasing PG and nonbilayer additives to di-18:1c-DGlcDAG (A) and di-18:1c-PC (B) bilayers, respectively. Increasing PG with bisPDPC probe (■); increasing PC in DGlcDAG with bisPDPC (●); increasing di-18:1c-PE in PC with bisPDPC (◇); increasing 1,3-DOG with bisPDPC (△); increasing 1,3-DOG (plus 10 mol % di-18:1c-MGlcDAG and 25 mol % di-18:1c-PG) in DGlcDAG with bisPDPG (▲ in A); increasing 1,3-DOG (plus 10 mol % di-18:1c-MGlcDAG and 35 mol % di-18:1c-PG) in PC with bisPDPG (▲ in B). The probe concentration was 0.1 mol %. For the DOG samples proper single bilayer liposomes (see Materials and Methods) could only be prepared at the DOG concentrations shown; at higher concentrations an L_α to nonlamellar phase transition took place (see text).

substrate (cf. above), dramatically increased the rate of DGlcDAG synthesis (Figure 1) but could not replace PG as an activator. Presence of the nonlamellar-prone di-18:1c-phosphatidylethanolamine (PE) in the PC bilayer stimulated DGlcDAG synthesis to some extent, but less than the DOG. *In vivo* the average amounts of PG is ≈20 mol % in di-18:1c enriched membranes (Wieslander et al., 1995), probably with an equal transmembrane distribution (Bevers et al., 1978), and the fraction of nonbilayer/nonlamellar-prone lipids in such membranes is 10−30 mol % (Wieslander et al., 1995).

These results strongly indicate that certain properties brought by the PG activator, nonbilayer additives, and bilayer matrix are important for the enzyme. Therefore, the lateral diffusion, potential domain formation, and chain ordering in single bilayer liposomes close to *A. laidlawii* size have been analyzed at experimental conditions crucial for the DGlcDAG synthase activity, using different pyrene chain-labeled lipids as probes.

Diffusion of PG in Different Bilayer Matrixes. The lateral diffusion and potential domain formation of PG was analyzed with PPDPG as probe (see Materials and Methods). Diffusion was slower in di-18:1c-DGlcDAG than in the corresponding PC matrix and increased especially in the former by increasing di-18:1c-PG fractions, according to the eximer/monomer fluorescence ratios obtained from the monopyrene chain probe (Figure 2). The diffusion rate for the PG probe was increased slightly in the absence of Mg^{2+} and in the presence of 10 mol % 1,3-DOG, respectively, but these differences were less than between the two bilayer matrixes in Figure 2. Diffusion of the matrix di-18:1c-PC was little affected by Mg^{2+} or PG but increased slightly by the presence of DOG (data not shown). These data revealed no indications for patch formation of PG since a local enrichment of these probe molecules to separate PG domains or phases should yield a substantially larger increase in the I_e/I_m ratios (Lehtonen & Kinnunen, 1995; Lehtonen et al., 1996).

It can be concluded that the translational diffusion of PG is slightly faster than for PC, the latter which is previously established. This may be explained from the smaller polar head of PG (cf. Vaz et al., 1984; van der Meer, 1993; Lindblom & Orädd, 1994). Consequently, PG diffusion in DGlcDAG is much more restricted by the larger disugar heads and perhaps also by intermolecular hydrogen bonds (cf. above). The opposite, i.e., faster diffusion in the presence of lipids with small heads like DOG, is then expected and also observed. An interaction with Mg^{2+} obviously lowers diffusion for the anionic PG but not for the zwitterionic PC. Electrostatic repulsion due to the charged PG headgroup should increase the spacing between lipids and enhance lateral diffusion. Mg^{2+} can partly neutralize anionic PG (Lau et al., 1981), allowing for a closer packing which should decrease the lateral diffusion, as observed. However, no domain formation of the essential PG activator and no obvious correlation between the diffusion rates and DGlcDAG synthase activity (as in Figure 1) were evident.

PG and 1,3-DOG Increase Acyl Chain Order. A potential factor for regulation of the integral (Dahlqvist, 1995) DGlcDAG synthase protein is bilayer chain order. In *A. laidlawii* membranes (and extracted lipids) a correlation between the MGlcDAG and DGlcDAG content and the average acyl chain order determined by NMR has been observed recently (Thurmond et al., 1994). Intramolecular excimer formation of bispyrene-chain lipid probes monitor free volume changes in bilayers (Lehtonen & Kinnunen, 1994); for the latter, a good theoretical and experimental correlation with acyl chain ordering can be inferred (Xiang, 1993). Different probes should report similar chain ordering in a given bilayer as long as the various lipids are homogenously distributed (cf. Thurmond & Lindblom, 1996).

According to the changes in I_e/I_m for the bisPDPC and -PG probes shown in Figure 3, membrane free volume is larger and the chain ordering thus lower in di-18:1c-DGlcDAG than in the corresponding PC. Stepwise addition of PC or PG to DGlcDAG eventually increased I_e/I_m ratios to the levels for the pure phospholipids. PG increased order in both matrixes, but more so in the DGlcDAG ones. A smaller increase was noted for increasing fractions of di-18:1c-PE in PC (Figure 3). Effects of DOG were substantial

even for small additions, especially in DGlcDAG bilayers. Most important, additive effects on chain ordering was caused by the simultaneous presence of PG and DOG in a bilayer matrix (Figure 3). Note that the range of I_e/I_m variation (hydrocarbon free volume) was substantially larger for most of the supplemented DGlcDAG matrixes, starting at much lower values, than for the PC ones. The increase in chain packing (i.e., I_e/I_m) by DOG supplements, as monitored by bisPDPG, was also dependent upon chain saturation and amounts of PG; more di-18:1c-PG present, or a 16:0−18:1c-PG species, yielded a slightly larger increase (data not shown). Proper liposomes or fluorescence signals could not be obtained above 25−30 mol % DOG, coinciding with the appearance of nonlamellar phases (see Materials and Methods, and below).

A good correlation is evident between the features of lipid packing in the single bilayer liposomes reported here by the bispyrene-chain probes (above) and acyl chain order and phase equilibria determined in bulk lipid samples by NMR and X-ray diffraction. The radius of spontaneous curvature for *A laidlawii* 18:1c DGlcDAG is slightly larger than the radius for di-18:1c-PC (Österberg et al., 1995). This can be explained by the larger (disugar) polar head of DGlcDAG and should yield a lower average acyl chain order due to more space for the two chains (Thurmond & Lindblom, 1996), as is visualized in Figure 3. Comparisons also indicate that the average chain order of DGlcDAG (Eriksson et al., 1991) is lower than the chain order for the corresponding PCs (Holte et al., 1995; Morrow et al., 1993). This may explain the large amounts of DGlcDAG synthesized and the correspondingly low order parameters obtained in *A. laidlawii* membranes *in vivo* with long acyl chains (≥18 carbons; Thurmond et al., 1994; Wieslander et al., 1995), where chain order perhaps otherwise would be prohibitively high. PE and especially DOG (cf. Figure 3 above) both increase chain order in PC matrixes according to NMR analyses (e.g., de Boeck & Zidovetzki, 1989; Goldberg et al., 1994; Lafleur et al., 1990). The increase obtained from PG in DGlcDAG or PC (Figure 3) is supported by the higher chain order parameter (obtained by NMR and FT-IR) for PG than for PC, and for anionic PG compared to the noncharged, protonated species (Tuchtenhagen et al., 1994). Important here is both the small PG polar head and the screening of its negative charge by the Mg^{2+} (cf. Lau et al., 1981), decreasing headgroup repulsion.

Hence, DOG, PG, or PE increases acyl chain order in DGlcDAG or PC bilayers in a concentration-dependent and additive manner, with stronger effects and larger range in a DGlcDAG matrix. A comparison between Figures 3 and 1 (plus results above) strongly indicates that this coincides with increases in DGlcDAG synthase activity.

Correlation between Chain Ordering, Phase Equilibria, and Enzyme Activity. To what extent can an order-increasing agent like 1,3-DOG increase DGlcDAG synthesis at physiological PG concentrations (≈20 mol % in 18:1c membranes)? Figure 4 reveals a close correspondence between the order increase (i.e., I_e/I_m ratios for bisPDPG) caused by DOG and the enzymatic synthesis of DGlcDAG (from MGlcDAG) in such bilayers. Note the somewhat higher order obtained with 25 mol % PG present (Figure 4). The strongly enhanced lipid synthesis peaked close to the order maxima and then decreased at higher DOG fractions (Figure 4). The enforced (frustrated) chain ordering in the bilayers,

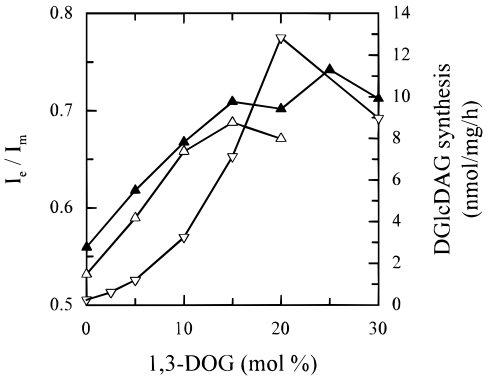

FIGURE 4: Correlation between chain ordering and DGlcDAG synthesis. I_e/I_m was recorded from bisPDPG at increasing 1,3-DOG concentrations in bilayers of 18:1c-DGlcDAG with 10 mol % 18:1c-MGlcDAG substrate, and 20 mol % (△) or 25 mol % (▲) di-18:1c-PG activator, respectively. Probe concentration was 0.1 mol %. Synthesis of DGlcDAG (▽) was analyzed at a suboptimal PG concentration, i.e., 20 mol % (cf. Figure 1), as in (△). Mean values from three experiments.

given by the decrease in interfacial molecular areas due to the 1,3-DOG present (Demel, 1994), should be released by a transition to nonbilayer (nonlamellar) aggregates of the reversed type (e.g., Lafleur et al., 1990). Such a transition is expected to start at 20−25 mol % DOG for the conditions used here, as extrapolated from some analogous systems (e.g., Goldberg et al., 1994; Nieva et al., 1995). This is supported by Figure 4, showing a reversal of the I_e/I_m ratios at these DOG concentrations, and by the inability to obtain proper liposomes with higher DOG fractions present (see Materials and Methods). The decrease in interfacial areas and the concomitant increase in chain order will increase the spontaneous curvature in these 18:1c lipids (Gruner, 1992). Chain order and enzyme activity decreased somewhat when the transition to nonbilayer aggregates of high curvature started, but activity remained fairly high (Figure 4).

In conclusion, an increase in chain order caused by low molar fractions of the nonbilayer-prone 1,3-DOG in bilayers of DGlcDAG strongly stimulated the DGlcDAG synthase activity in a correlated manner at suboptimal (physiological) concentrations of the essential activator lipid PG.

Local Enrichment of PG by Chain Length Mismatch. Since the enzyme was very responsive to the PG concentration (Figure 1), any occurrence of PG-enriched domains to which the DGlcDAG synthase has access should promote enzyme activity. However, even at conditions of increased chain order, spontaneous curvature, and enzyme activity (above and Figure 4), there were no obvious indications of a local enrichment of the PG activator. Gel phase domains of PG in liquid bilayers cannot stimulate the enzyme, which seems to demand a liquid-crystalline activator PG (Dahlqvist et al., 1995).

Figure 5A shows the effects of a chain length mismatch between di-18:1c-PG and different unsaturated PCs. A decrease in chain length for the PC matrix by four carbons, i.e., from 18 to 14 carbons, yielded a larger change in I_e/I_m as reported by PPDPG than did an increase by four carbons (from 18 to 22 carbons). The maximal change for the 14:1c-PC sample was larger than for any other PC matrix investigated by monopyrene-chain probes here (Figure 2 and data not shown). Evidence for the formation of PC microdomains in a short-chain PC matrix under similar conditions of length mismatch was recently found with a combination

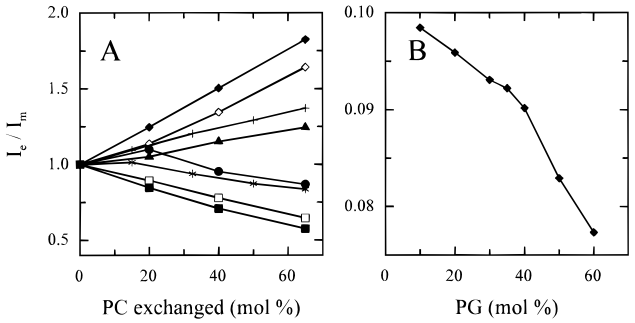

FIGURE 5: Behavior of di-18:1c-PG in phosphatidylcholine bilayers with different acyl chains. (A) I_e/I_m was monitored: for PPDPG (1 mol %) in liposomes made with a constant fraction di-18:1c-PG (35 mol %) plus di-18:1c-PC (control mixture), where the latter was exchanged stepwise to di-14:1c-PC (◆), di-16:1c-PC (▲), di-20:1c-PC (●), and di-22:1c-PC (■), respectively; likewise for PPDPC (1 mol %) from control to di-14:1c-PC (◇) and di-22:1c-PC (□); and for bisPDPG (0.1 mol %) in 18:1c control and bilayers enriched in di-14:1c-PC (+) and di-22:1c-PC (*). Changes in relative I_e/I_m, normalized to starting conditions, are shown as a function of the mol % PC species exchanged in the liposome bilayers. (B) Increasing fractions di-18:1c-PG in di-14:1c-PC, monitored by I_e/I_m from PPDPG (1 mol %).

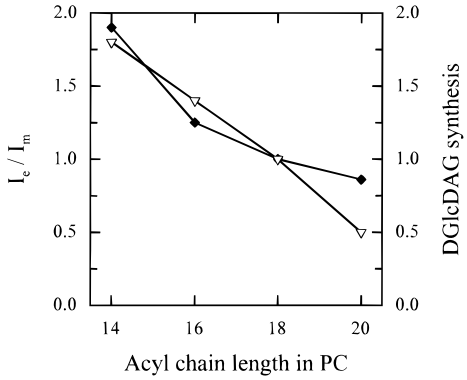

FIGURE 6: DGlcDAG synthesis in PC bilayers of different thickness. The rates of DGlcDAG synthesis (▽) were assayed in bilayers containing 40 mol % di-18:1c-PG activator, 10 mol % MGlcDAG substrate, and 50 mol % of either di-14:1c-, di-16:1c-, di-18:1c-, or di-20:1c-PC, respectively. Mean values are from three experiments, normalized to the values for the 18:1c samples. An equally good correlation was obtained if the bilayer thickness (in nm) for pure PCs (Lewis & Engelman, 1983) was plotted on the *x*-axis. The maximum relative I_e/I_m values (◆) reported by PPDPG for the corresponding PC bilayers in Figure 5A are shown for comparison.

of techniques (Lehtonen et al., 1996). In brief, the PPDPC probe was shown to be dispersed or clustered as a function of matrix chain length; this was supported by analysis of chain dynamics and by resonance energy transfer measurements with another, colocalized probe (Lehtonen et al., 1996). The changes in I_e/I_m ratios for the corresponding PG/PC matrixes here (Figure 5) were slightly larger than for that system. It is well established that lipid lateral diffusion is faster with shorter chains, but the type of polar headgroups often have a larger impact (Vaz et al., 1984; van der Meer, 1993; Lindblom & Orädd, 1994; see also Figure 2). Even if two components have a fluid−fluid miscibility, they need not behave identically in mixtures, e.g., in a binary mixture the average acyl chain order for a di-18:0-PC (25 mol %) is substantially higher than for the corresponding di-14:0-PC matrix (75 mol %) (Lu et al., 1995). Theoretical analyses indicate that these two PCs experience nonideal mixing, with formation of small domains in the liquid-crystalline phase, due to the chain length mismatch (Jørgensen et al., 1993). Most important, the mixing behavior of the two lipids in single bilayers are affected by liposome (curvature) diameters (Brumm et al., 1996), within the size range for *A. laidlawii* cells and the model systems used here. Nonideal mixing may be enhanced if the participating lipids have different polar headgroups, as observed for the anionic phosphatidylserine (PS) in PC (Huang et al., 1993). It is therefore very likely that the substantial increase in I_e/I_m for PPDPG, from the 18:1c to the 14:1c PC matrix (Figure 5A), was caused by patch formation and an enhanced rate of diffusion, whereas the smaller change in I_e/I_m from the 18:1c to the 22:1c matrix was determined by diffusion alone. Strong support for this interpretation is given by Figure 5B, showing increased I_e/I_m ratios for the PG probe with decreasing 18:1c-PG concentration in a 14:1c-PC matrix. This indicates an extensive patching of PG even at low concentrations. The increase in I_e/I_m is probably caused by an enrichment of the probe in smaller or fewer PG domains with decreasing PG concentrations. Note that the I_e/I_m ratios for the corresponding PC probe were smaller (Figure 5A) and similar to a PC/PC mismatch matrix (Lehtonen et al., 1996), indicating a lesser influence of the chain variations for the behavior of PC than for PG, in a PC matrix.

Acyl chain ordering, as monitored by bisPDPG (of similar length as the 18:1c lipids), was increased in a thin but not in a thick PC matrix (Figure 5A). Normally, chain ordering is lower in a thinner compared to a thicker bilayer matrix, as monitored by NMR (Lu et al., 1995).The increase here (Figure 5A) was of similar magnitude to the order increase in an analogous PC/PC mismatch matrix (Lehtonen et al., 1996) and also similar to that brought by DOG or by a substantial increase in the PG concentration in DGlcDAG bilayers, (cf. Figures 3 and 4). Such a response is expected from a probe participated to local domains of di-181c-PG forced together in a thinner bilayer lipid environment.

Hence, for di-18:1c-PG the lateral diffusion is increased, and local enrichment into domains is enhanced by a fluid PC bilayer with shorter, but not longer, acyl chains.

Chain Length Mismatch Stimulates DGlcDAG Synthase Activity. There was no correlation between the I_e/I_m ratios for PPDPG, reflecting the lateral diffusion of PG, and enzyme activities in the PC and DGlcDAG matrixes as shown in Figures 1 and 2. However, a local enrichment of di-18:1c-PG, as indicated by Figure 5, should stimulate the DGlcDAG synthase. This prediction was substantiated, and increased rates of synthesis in short-chain PC matrixes were evident (see Figure 6). The 22:1c-PC species was not used here because of its higher melting temperature, and the di-18:1c-PG activator was kept at an efficient 40 mol % level (cf. Figure 1, PC-matrix). Bilayers also contained 10 mol % 18:1c-MGlcDAG substrate which may partition with the di-18:1c-PG of equal length, but *in vitro* the DGlcDAG synthase is little affected by variation of substrate concentration in this range (Dahlqvist et al., 1995). *In vivo* thinner membranes yield much less DGlcDAG (and more MGlcDAG) synthesized than thicker ones, in agreement with the spontaneous curvature and phase equilibria valid for thin bilayers (Thurmond et al., 1994; Wieslander et al., 1995). Here, a good correlation for the synthesis rates with the I_e/I_m ratios from the PPDPG probe (Figure 6) strongly indicates that a local enrichment of the 18:1c-PG activator in a short-chain

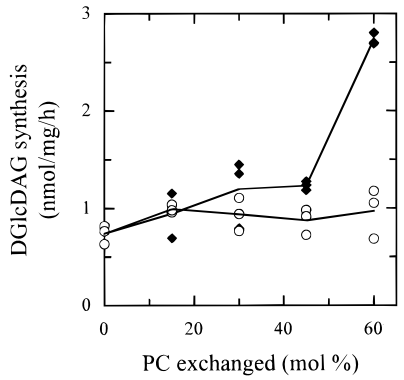

FIGURE 7: Stimulation of DGlcDAG synthesis by chain length mismatch. The rates of DGlcDAG synthesis were assayed in bilayers containing 30 mol % di-18:1c-PG activator, 10 mol % MGlcDAG substrate, and increasing fractions of di-14:1c-PC (◆) or di-20:1c-PC (○), in a matrix of di-18:1c-PC. 30 mol % di-18:1c-PG yielded a low, suboptimal enzyme activity in a PC matrix (see Figure 1).

PC environment stimulated the enzyme, overriding the inhibitory effects of curvature on enzyme activity occurring in thin membranes.

The difference in rates of synthesis between the thinnest and the thickest matrix was approximately 4-fold at maximal PC fractions exchanged and at an efficient activator concentration (see Figure 6). To what extent can the enzyme activity be enhanced at a suboptimal PG concentration? Figure 7 reveals that at a di-18:1c-PG content of 30 mol % (cf. Figure 1, PC matrix) the fraction of the short PC species must be above 45% in the liposome bilayer before a substantial stimulation of enzyme activity occurred, eventually reaching a 4-fold enhancement at the maximum level of PC exchanged. This range of di-14:1c-PC corresponded to conditions of patching for di-18:1c-PG, according to Figure 5 and the discussion above. Note the absense of a potentiation of enzyme activity in the 20:1c matrix, corroborating the diffusion data.

A lateral redistribution of PG due to protein−lipid interactions or gel phase formation has been observed in *A. laidlawii* membranes *in situ* (Bevers et al., 1978, 1979). The experiments performed here have revealed that a lateral enrichment of PG into domains, due to a chain length mismatch in fluid liposome membranes, can stimulate the activity of DGlcDAG synthase both at efficient and suboptimal average bilayer concentrations of the essential activator PG.

CONCLUSION

So far, the physicochemical properties of the *A. laidlawii* membrane lipids have been determined mainly in bulk samples at high concentration. However, the metabolic setting of the packing constraints is achieved by the different lipid-synthesizing enzymes in single bilayers at low concentration. We have therefore sought a more cell-like system for the analysis of lipid properties concommitant with enzyme activities. The results presented here reveal that pyrene-labeled lipid probes can be used at very low lipid concentrations to monitor lipid dynamics in liposome bilayers and that the recorded signals can be correlated to changes in activity of the *A. laidlawii* DGlcDAG lipid synthase. Furthermore, the size of the liposomes were close to that of this small bacterium, lending further credence to the experimental approach.

Lateral Diffusion and Domain Formation of the Essential Activator Lipid. Phosphatidylglycerol is probably the most common polar membrane lipid among prokaryotes, indicating a conserved biosynthetic pathway (Ratledge & Wilkinson, 1988). In *A. laidlawii* it is needed in large amounts as an essential activator of the DGlcDAG lipid synthase in the other, glucolipid pathway (Figure 1). The translational diffusion of di-18:1c-PG was slower in di-18:1c-DGlcDAG bilayers compared to PC ones, perhaps due to hydrogen bonding between the former two, but local domains with higher PG concentrations were not formed at conditions of high DGlcDAG enzyme activity (Figures 1 and 2). Furthermore, there was no correlation between the measured I_e/I_m values (correlating to the rates of lateral diffusion) for the PG activator and DGlcDAG synthase activity, although diffusion was slightly increased by certain activity-promoting lipids like PG and 1,3-DOG (Figure 2). The latter two both increased chain order (Figure 3), and DOG also increase the spontaneous curvature. Hence, it can be concluded that a stimulation of the DGlcDAG synthase activity by an increased PG concentration, acyl chain order, or spontaneous curvature, respectively, is not occurring as a function of the diffusion rate or domain formation by the essential activator lipid PG.

The synthesis of DGlcDAG *in vivo* is normally low in membranes with short acyl chains, since these are more lamellar-prone with a lower spontaneous curvature. PG fractions are also small in such bilayers (Wieslander et al., 1995). A chain length mismatch between a di-18:1c-PG and a thinner, monounsaturated PC matrix caused a local concentration- and matrix-dependent enrichment of PG (Figure 5), sufficient to stimulate the DGlcDAG synthase in a correlated manner, even at lower (suboptimal) PG fractions (Figures 6 and 7). An MGlcDAG substrate (10 mol %) of equal chain length may partition with the PG, while the DGlcDAG synthase is little responsive to substrate concentration in relation to the effects of curvature (Dahlqvist et al., 1995). Domain formation under these conditions of chain length mismatch is expected both on theoretical (Jørgensen et al., 1993) and experimental grounds (Huang et al., 1993; Lehtonen et al., 1996), but their lifetimes are not known. In *A. laidlawii*, large differences in acyl chain length can occur (Wieslander et al., 1995), and PG may have a heterogenous lateral distribution (Bevers et al., 1978, 1979). Hence, a local enrichment of PG (or chain variants thereof) by chain mismatch is a possible mechanism to influence the activity of the DGlcDAG synthase *in vivo*, which potentially may supersede substrate acyl chain preferences of the enzyme (Dahlqvist et al., 1995) or curvature conditions normally valid in the bilayer (Österberg et al., 1995). This makes rapid enzymatic adjustments in MGlcDAG and DGlcDAG amounts possible, by a directed lateral displacement of the PG activator.

Curvature Is Crucial for DGlcDAG Synthase Activity. A correlation between increases in chain order and a concommitant increase in DGlcDAG synthase activity is clearly evident in these experimental systems (compare Figures 1, 3, and 4). In *A. laidlawii* membranes the spontaneous curvature (Österberg et al., 1995) and the phase equilibria (Lindblom et al., 1986) are kept fairly constant for the lipids with different acyl chains, as analyzed for extracted lipids with X-ray diffraction and NMR, due to metabolic adjustments between especially MGlcDAG and DGlcDAG. How-

ever, the average chain order is substantially higher in thin membranes by the metabolical enrichment of MGlcDAG (small head), compared to the lower order in thick membranes, enriched in DGlcDAG (larger head) (Thurmond et al., 1994). Likewise, cholesterol and certain solvents stimulate DGlcDAG synthesis; the former increases chain order, the latter decreases it due to a different membrane localization (Wieslander et al., 1986), but both increase the spontaneous curvature (Gruner, 1992). The increase in chain order and curvature brought by DOG in DGlcDAG (Figure 4), as deduced from the established behavior of DOG in several other lipids (e.g., Goldberg et al., 1994), is thus counteracted by the stimulated synthase producing more DGlcDAG which lower chain order (Thurmond et al., 1994) and curvature (Österberg et al., 1995) (Figure 3). A comparison between Figures 1 and 3 reveals that DGlcDAG synthesis was higher in a DGlcDAG compared to PC matrix but chain order (i.e., I_e/I_m) was lower; an increase in order to a level similar in PC, by increasing PG or introduction of DOG, enhanced activity further in the DGlcDAG matrix. These additives had analogous effects in the PC matrix, but with a smaller range. This strongly indicates that it is not the absolute values of the chain order that governs the activity of the DGlcDAG synthase, but rather changes in the actual spontaneous curvature (brought by the additives) within a certain range of properties where responsiveness is optimal. Furthermore, the enzyme may also have a more specific or productive interaction with the DGlcDAG matrix. Such specificities are known for several other proteins, e.g., the chloroplast ferredoxin precursor have higher specificities toward PG and several monogalactosyl-DAG species (Demel et al., 1995). However, the activity of the *A. laidlawii* DGlcDAG synthase in an unsaturated DGlcDAG or PC matrix, respectively, clearly follows the anticipated spontaneous curvature, as modulated here by established order-increasing, nonbilayer-prone agents.

The substantial amounts of PG needed to activate the DGlcDAG synthase indicates that bilayer properties must be changed or that several sites for binding or interaction with PG must be saturated (cf. Newton, 1993), perhaps in a cooperative fashion (Karlsson et al., 1994). This can only be achieved by the presence of a large overall or domain concentration of PG (cf. above). The proposed integral nature of the enzyme (Dahlqvist, 1995) makes a conformational change of the protein more likely than an electrostatic binding to the membrane surface. Conformation of the analogous monogalactosyl-DAG synthase from chloroplasts is affected by the presence of PG (Maréchal et al., 1995). The curvature stress in a bilayer close to an L_α to nonlamellar phase transition (like in Figure 4) has a magnitude that may influence protein conformation (Gruner, 1994). It can be proposed here that this packing strain (i) facilitates a conformational activation of the *A. laidlawii* DGlcDAG synthase at subsaturation levels of PG perhaps by exposing activation sites on the enzyme more efficiently for PG; or (ii) affects physical properties of PG and/or the bilayer matrix, important for PG/DGlcDAG synthase contacts.

ACKNOWLEDGMENT

We thank Viola Tegman and Birgitta Rantala for technical assistance and Jukka Lehtonen and Lu Li for discussions.

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BI9602876