Efficient Modulation of Glucolipid Enzyme Activities in Membranes of *Acholeplasma laidlawii* by the Type of Lipids in the Bilayer Matrix†

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ABSTRACT: It is generally anticipated, but so far not fully shown, that the physical properties of membrane lipid bilayers are governed by the concerted actions of the lipid-synthesizing enzymes. In the membrane of *Acholeplasma laidlawii* a constant surface charge density, similar phase equilibria, and a nearly constant spontaneous curvature are maintained for the polar lipids. Important for these properties are monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), forming mainly reversed nonlamellar and lamellar phases, respectively. The syntheses of these lipids (from 1,2-DAG and MGlcDAG) by two consecutively acting, membrane-bound glucosyltransferases have been analyzed in synthetic lipid bilayers of selected physical properties. Both enzymes demanded the presence of activator lipids; for MGlcDAG synthesis a critical fraction of anionic lipids was important, whereas for the DGlcDAG synthesis substantial amounts of a liquid-crystalline phosphatidylglycerol (PG) with a certain chain length were essential. The rates of the syntheses for the two glucolipids increased with decreasing chain length of the DAG and MGlcDAG substrates. The enzymatic formation of DGlcDAG (bilayer-forming) was influenced in a dose-dependent manner by the nonbilayer (curvature) propensities of several amphiphilic and hydrophobic lipids in two different bilayer matrixes. However, the preceding synthesis of the nonlamellar MGlcDAG was only affected to a minor extent by such additives. The mechanism for modulation involved an enhancement of the activating potencies of PG in a cooperative fashion at physiological concentrations for PG. The effects of substrate acyl chain properties, PG activator amounts, and nonbilayer additives on the syntheses of MGlcDAG and DGlcDAG *in vitro*, are sufficient *for* and in agreement *with* the metabolic changes in molar fractions of these two glucolipids and PG occurring under a variety of conditions *in vivo*.

Many membrane-bound proteins are dependent upon specific lipids or lipid-associated conditions for optimal function (Bienvenüe & Sainte-Marie, 1994; Newton, 1993). The composition and physical properties of the lipid bilayers in biological membranes are determined by concerted actions of the individual, often membrane-bound enzymes synthesizing the different lipids (Hjelmstad & Bell, 1991). However, the nature of the properties maintained for the lipids under different environmental conditions are so far only partially defined (Hazel & Williams, 1991).

The small cell-wall-less, parasitic bacterium (mycoplasma) *Acholeplasma laidlawii* can only modify the crucial gel to liquid crystalline (L_β to L_α)[1] phase transition temperature (T_m) of the membrane lipids to a minor extent, since it can only synthesize saturated but not unsaturated fatty acyl chains which are essential for survival [reviewed in McElhaney (1992)]. However, the syntheses *in vivo* of all different major lipids in the bilayer are strongly adjusted in a characteristic manner by factors or conditions that effect the abilities of the individual lipids to pack into bilayer or nonbilayer aggregates *in vitro* [reviewed in Rilfors et al. (1993) and Wieslander (1995)]. Thereby, a potential transition from a lamellar (L_α) to a nonlamellar (reversed cubic, Q_{II}/reversed hexagonal, H_{II}) phase (Rilfors et al., 1993), and a certain negative spontaneous curvature (Österberg et al., 1995), are maintained fairly constant for the bilayer lipid mixtures under a variety of conditions *in vivo*. This is also the case for the surface charge density of the anionic phospholipids in the bilayer (Christiansson et al., 1985; Wieslander et al., 1995).

The polar lipids are all made from a minor phosphatidic acid (PA) precursor in two competing pathways: (1) one leading to the major phosphatide phosphatidylglycerol (PG)

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[1] Abbreviations: lipid acyl chains, 8:0, octanoyl; 12:0, dodecanoyl; 14:0, tetradecanoyl; 15:0, pentadecanoyl; 16:0, hexadecanoyl; 17:0, heptadecanoyl; 18:0, octadecanoyl; 19:0, nonadecanoyl; 20:0, eicosanoyl; 14:1c, tetradecenoyl (9*cis*); 16:1c, hexadecenoyl (9*cis*); 18:1c, octadecenoyl (9*cis*) (or oleoyl); 18:2c, octadecedienoyl (9*cis*, 12*cis*); 20:1c, eicosenoyl (11*cis*); CHM, cyclohexylmethyl; DAG, 1,2-diacylglycerol; DGlcDAG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-(*O*-α-D-glucopyranosyl)]-*sn*-glycerol; Glc, glucose; GPDGlcDAG, 1,2-diacyl-3-*O*-[glycero-3-phospho-6-*O*-α-D-glucopyranosyl-(1→2)-(*O*-α-D-glucopyranosyl)]-*sn*-glycerol; MGlcDAG, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, PG-3-phosphate; TLC, thin-layer chromatography; H_{II}, reversed hexagonal, liquid-crystalline phase; L_α, lamellar liquid-crystalline phase; L_β, lamellar gel phase; Q_{II}, reversed cubic, liquid-crystalline phase; T_m, L_β to L_α phase transition temperature.

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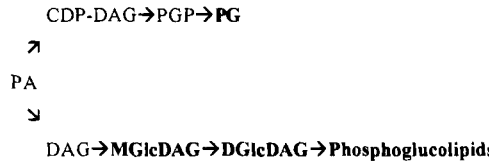
and (2) the other by way of the minor 1,2-diacylglycerol (DAG) sequentially to the major glucolipids monoglucosyl-DAG (MGlcDAG) and diglucosyl-DAG (DGlcDAG) and then to two phosphoglucolipids, respectively (Rilfors et al., 1993).



MGlcDAG (and DAG) form mainly nonlamellar (nonbilayer) phases, whereas DGlcDAG and PG form lamellar phases only (Lindblom et al., 1986, 1993). Under certain conditions DAG and an acylated (nonbilayer) derivative of MGlcDAG may accumulate (Wieslander et al., 1995). Two different, membrane-bound glucosyltransferases catalyze the consecutive reactions: (i) DAG + UDP-Glc → MGlcDAG + UDP and (ii) MGlcDAG + UDP-Glc → DGlcDAG + UDP (Smith, 1969; Dahlqvist et al., 1992; Dahlqvist, 1995). *In vivo* the molar ratio of MGlcDAG/DGlcDAG is decreased by factors that shift the phase equilibria from L_α phases toward H_{II} and Q_{II} phases (Rilfors et al., 1993; Wieslander, 1995). Such factors include acyl chain length and unsaturation, growth temperature, and incorporation of solvents, detergents, and steroids into the membrane. The PG content usually varies in direct relation with DGlcDAG (Wieslander et al., 1995); however, unlike the glucolipids the response of the PG content to new conditions is not rapid. The amounts of the two phosphoglucolipids are larger in thin *A. laidlawii* bilayers, i.e., bilayers that contain short acyl chains (Thurmond et al., 1994a; Wieslander et al., 1995), but molar amounts of polar headgroup phosphates are kept fairly contstant. *In vitro* the syntheses of MGlcDAG and DGlcDAG are dependent upon the presence of a certain surface charge density of anionic phospholipids and upon critical and substantial amounts of an activator lipid (especially PG), respectively. The type of bilayer matrix lipid is also important (Karlsson et al., 1994).

The mechanism by which the balance between MGlcDAG (nonbilayer-forming) and DGlcDAG (bilayer-forming) and thereby certain lipid-packing properties of the membranes (above) are sustained is possibly (i) by regulation at the level of gene transcription and enzyme expression or (ii) by modulation of the activities of the two glucosyltransferases. Lipid degradation is probably not involved [cf. Christiansson and Wieslander (1978), Dahlqvist et al. (1992), and Nyström et al. (1992)]. Rapid shift experiments, where significant changes in the glucolipid composition are obtained within a short time (Clementz et al., 1986; Wieslander et al., 1986), make the latter of the two alternatives more likely. It is therefore proposed that the structural or dynamic features of the membranes are the properties that are sensed by conformational changes of the lipid-synthesizing enzymes.

Here, the impact of (i) activator concentrations and properties, (ii) chain length and unsaturation of the glucolipid precursors, and (iii) the presence of bilayer-packing perturbants with varying propensities on the syntheses of MGlcDAG and DGlcDAG has been examined in bilayer model systems. It is found that the effects of activator and bilayer matrix lipids in particular on the enzymatic synthesis of MGlcDAG and DGlcDAG *in vitro* are sufficient *for* and in accordance *with* the extensive variations in amounts of these two lipids observed under a variety of conditions *in vivo*.

MATERIALS AND METHODS

Organisms and Growth Conditions. *A. laidlawii* strain A-EF22 was grown at 28 °C in a tryptose/bovine serum albumin medium (Dahlqvist et al., 1992). The medium was supplemented with 0.12 mM octadecenoic (oleic) acid (18:1c) and 125 μCi per liter of an [^{3}H]-labeled amino acid mixture (Amersham International, Solna, Sweden). The cells were harvested by centrifugation, washed twice (Dahlqvist et al., 1992), and stored in 0.2 M Tris−maleate buffer, pH 8.0 (≈20 mg protein/mL), at −80 °C. The protein content was determined according to Minamide and Bamburg (1990).

Substrate and Matrix Lipids. All the different diacylglycerol and dialkylglycerol (DAG) species were purchased from Larodan (Malmö, Sweden). Phosphatidylglycerols (Na^+ form) and phosphatidylcholines (PC) were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Di-16:0-phosphatidylcholine with a cyclohexylmethyl group substituted to carbon 2 of each acyl chain (CHM-di-16:0-PC) was provided by Dr. Ruthven Lewis (Lewis et al., 1994). The poly(oxyethylene) alkyl ether detergent $C_{16}EO_8$ and the sterols and steroids were purchased from Sigma (St. Louis, MO, U.S.A.). Unsaturated MGlcDAG and DGlcDAG were prepared from *A. laidlawii* grown in presence of oleic acid. Lipids (0.2 g) extracted from such cells (Ericsson et al., 1991) were applied on a silicic acid (Bio-Sil HA-325 mesh, Bio-Rad Laboratories) column (diameter 35 mm/height 15 mm) dissolved in 50 mL of chloroform. Pigments and neutral lipids were eluted with chloroform (5 × 10 mL), monoacyl-MGlcDAG with chloroform/methanol (98:2 v/v; 5 × 10 mL), MGlcDAG with chloroform/methanol (95:5 v/v; 5 × 10 mL), DGlcDAG with chloroform/methanol (9:1 v/v; 5 × 10 mL), and the remaining lipids with pure methanol. By separating the MGlcDAG and DGlcDAG fractions a second time, a purity of ≥95% as judged from thin-layer chromatography (TLC) was obtained for the lipids. More than 90% (mol/mol) of the acyl chain content of MGlcDAG and DGlcDAG was 18:1c chains according to GLC analysis (Wieslander et al., 1995). The remaining fraction was mainly 16:0 and eicosenoyl chains (20:1c). Synthetic, saturated, and branched-chain MGlcDAG was synthesized, purified, and characterized as described earlier (Mannock et al., 1990b).

Assay for MGlcDAG and DGlcDAG Synthesis. The enzymatic synthesis of MGlcDAG and DGlcDAG was analyzed with lipid-depleted membranes prepared by solvent extraction of lyophilized cells (Karlsson et al., 1994). Extraction with pure acetone followed by acetone/methanol 9:1 (v/v) removed 96% of the membrane lipids. This procedure with a subsequent lipid supplementation enhanced the synthesis rate of MGlcDAG and DGlcDAG to 55 and 25 nmol/(mg of cell protein) h^{-1}, respectively, as compared with 5 and 20 nmol/(mg of cell protein) h^{-1} for nonextracted cells (Dahlqvist et al., 1994). Selected lipids dissolved in benzene were added to 0.5 mg aliquots of depleted membranes (Karlsson et al., 1994). The benzene was evaporated under a stream of N_2, leaving the lipids (0.5 mmol/g of depleted cells) in direct contact with the proteins. After incubation of the dry mixture at 4−8 °C for 30 min, 80 μL of 0.15 M Tris−maleate buffer (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, and the mixtures were incubated for 10 min at 28 °C. The syntheses were stopped by addition of 375 μL of methanol/chloroform 2:1 (v/v). Ultracentrifugation of reconstituted samples in Percoll gradients showed that over 80% of the added lipids were recovered in a low-density fraction mixed with a fairly constant fraction of the proteins, dominated by membrane proteins (Karlsson et al., 1994). The lipid compositions in these fractions were identical to the ones in the original mixtures.

The enzymatically synthesized MGlcDAG and/or DGlcDAG were extracted and separated on TLC plates as described by Karlsson et al. (1994) and then quantified using electronic autoradiography (Packard Instant Imager). The synthesis rates of MGlcDAG and DGlcDAG were expressed as nanomoles of lipid synthesized per milligram of protein per hour from the amount of glucose incorporated into the lipids (i.e., MGlcDAG from DAG, and DGlcDAG from MGlcDAG). For MGlcDAG the total amount of glucolipids synthesized (MGlcDAG plus DGlcDAG) was taken into account, since newly synthesized MGlcDAG could be further processed to DGlcDAG.

RESULTS AND DISCUSSION

The syntheses of MGlcDAG and DGlcDAG have been analyzed in lipid bilayers of selected physical properties. Important features regarding the physiological regulation of these lipids have been included in the model systems. These are (i) the chain length and unsaturation of the essential PG activator, (ii) the chain length (bilayer thickness) of the bilayer matrix, (iii) the chain length and unsaturation of the lipid precursor substrates, and (iv) the presence of different nonlamellar and nonbilayer-preferring foreign molecules with varying but established propensities to affect the equilibria between L_α and nonlamellar phases for membrane polar lipids.

Activation of Glucolipid Synthesis by Various Phosphatidylglycerols. The stimulation of DGlcDAG synthesis *in vitro* by PG follows apparent cooperative kinetics (Karlsson et al., 1994). Synthesis of MGlcDAG seemed more dependent upon proper surface charge density and potential given by anionic lipids.

Table 1 reveals that the chain properties of the activator lipid PG are also important. Di-14:0-, di-18:0-, or di-18:1c-PG was used at maximum activating concentrations [cf. Karlsson et al. (1994)]. These PGs have T_m of approximately 24, 55, and −18 °C, respectively (Findlay & Barton, 1978), whereas T_m for di-18:1c-DGlcDAG is approximately −20 °C (Lindblom et al., 1986). The most efficient activation was achieved with di-18:1c-PG. Saturated acyl chains of 18 carbons were not able to activate the synthesis of the glucolipids, while synthesis of not only MGlcDAG but also DGlcDAG could be detected when a PG with short chains (i.e., 14:0) was added to a concentration of at least 30 mol % (data not shown). The high melting temperature of di-18:0-PG will most likely prevent its full miscibility at 40 mol % with the liquid-crystalline phase of the matrix. Although di-14:0-PG will be in the liquid-crystalline phase at the assay temperature (28 °C), it could only partially activate the two glucolipid-synthesizing enzymes. Di-18:0-PG combined with di-18:1c-PG could fully activate the MGlcDAG synthesis similar to the activity gained with di-18:1c-PG only. For the synthesis of DGlcDAG none of the saturated PGs tested could equally substitute di-18:1c-PG. A change from 18:1c to saturated chains in the matrix lipids had a substantially smaller influence than the change of activator chains (cf. Table 1) for the MGlcDAG synthesis (data not shown).

Table 1: Glucolipid Synthesis with Different Acyl Chains in the Phosphatidylglycerol Activator and the Bilayer Matrix[a]

acyl chain species of PG activator	glucolipid synthesis	
	MGlcDAG	DGlcDAG
di-14:0	0.39 ± 0.03	0.11 ± 0.03
di-18:0	0.02 ± 0.02	0.02 ± 0.03
di-18:1c	1.00 ± 0.05	1.00 ± 0.14
di-14:0 + di-18:1c	0.61 ± 0.06	0.55 ± 0.18
di-18:0 + di-18:1c	0.97 ± 0.15	0.32 ± 0.10
acyl chain species in PC matrix	**glucolipid synthesis**	
	MGlcDAG	DGlcDAG
di-14:1c	0.67 ± 0.04	1.78± 0.25
di-16:1c	1.09 ± 0.19	1.45 ± 0.24
di-18:1c	1.00 ± 0.07	1.00 ± 0.15
di-20:1c	0.91 ± 0.09	0.47 ± 0.04

[a] The rates of synthesis were assayed with 40% or 20% + 20% (mol/mol) of PG with different acyl chains in a matrix of di-18:1c-DGlcDAG, containing 5 mol % di-18:1c-DAG or 10 mol % di-18:1c-MGlcDAG substrates, respectively. Matrix chain length was varied using PC with different unsaturated acyl chains and di-18:1c-PG as the activator. The data are mean values from three experiments, normalized to the values determined with 18:1c-chains (corresponding respectively to approximately 37 and 9 nmol of MGlcDAG or DGlcDAG synthesized/(mg of cell protein) h^{-1}.

These experiments revealed that (i) the activator lipid must be in a liquid-crystalline state in order to promote the syntheses of the two glucolipids and (ii) the extent of activation of the DGlcDAG synthesis was more sensitive to the chain length and unsaturation of the PG activator as compared to the MGlcDAG synthesis.

Bilayer Thickness Affects Activation. Table 1 (lower part) reveals that for lipid substrates and activator PG of identical chain structure (i.e., di-18:1c), the synthesis of DGlcDAG but not that of MGlcDAG was substantially affected by the matrix chain length. For phosphatidylcholines (PCs) as well as for *A. laidlawii* lipids, such acyl chains yield substantial differences in lipid bilayer thickness (Lewis & Engelman, 1983; Thurmond et al., 1994a). Note the correlation between decreasing matrix chain length and increasing DGlcDAG synthesis (Table 1). *In vivo* more DGlcDAG is synthesized with increasing bilayer thickness (Wieslander et al., 1995), opposite to what was shown *in vitro* (Table 1). Liquid-crystalline PC molecules differing in acyl chain length by four carbons can form partially separate, local domains in the fluid phase (Jørgensen et al., 1993). This may also apply to PG in PC. Studies using fluorescent-labeled PG have indicated that the extent of patching for PG follows closely the DGlcDAG synthase activity and matrix chain length in Table 1, lower part (Karlsson et al., manuscript in preparation). Hence, locally concentrated PG molecules in thinner bilayers (Table 1) would be beneficial for activation of the DGlcDAG synthesis, perhaps through binding to the enzyme, but not affecting the MGlcDAG synthase.

It can be concluded that in unsaturated bilayers differences in chain length between the matrix lipid and a standard PG activator have a strong impact especially on the synthesis of DGlcDAG, probably through local enrichment of PG activator molecules.

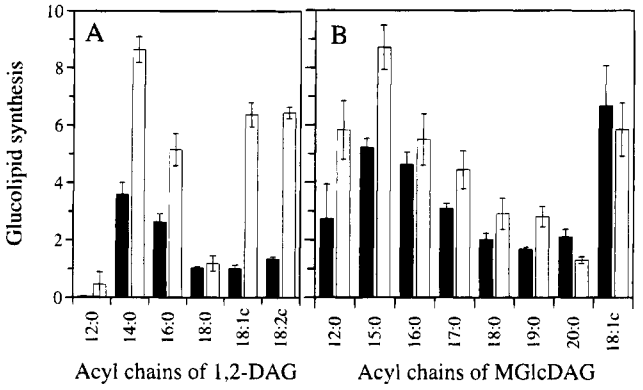


FIGURE 1: Acyl chain variants of lipid substrates. The rates of MGlcDAG synthesis (A) were assayed with 5 mol % (solid bars) or 30 mol % (open bars) of various 1,2-DAG substrates, in a matrix consisting of di-18:1c-PG (50 mol %) and di-18:1c-PC. The rates of synthesis are normalized to the values obtained in presence of 5 mol % di-18:1c-DAG. DGlcDAG synthesis (B) was assayed as above with 10 mol % (solid bars) or 40 mol % (open bars) of various MGlcDAG substrates. The rates are normalized to the values obtained in presence of 10 mol % di-18:1c-MGlcDAG (cf. Table 1). Bars are standard error of mean value for three experiments.

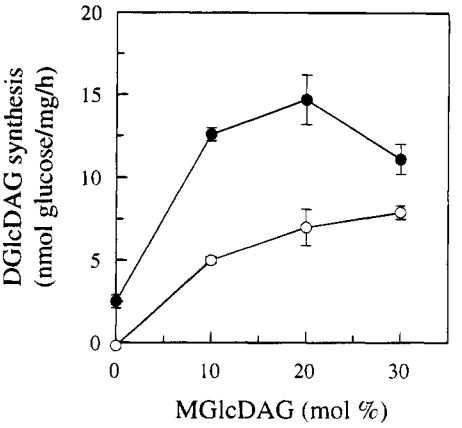


FIGURE 2: Stimulation of DGlcDAG synthesis by DAG. The synthesis of DGlcDAG was assayed with increasing amounts of di-18:1c-MGlcDAG substrate (○), together with 5 mol % 1,2-di-18:1c-DAG (●), in a lipid matrix of di-18:1c-PG (30 mol %) and di-18:1c-DGlcDAG. The fraction of MGlcDAG was enzymatically increased by less than 1 mol % of the total lipids during the assay. Rates as nmoles of glucose incorporated into DGlcDAG per milligram of protein per hour (mean values from three experiments). Error bars as in Figure 1.

Acyl Chain Variants of the Lipid Substrates. Synthetic species of the lipid substrates 1,2-DAG or MGlcDAG, with saturated acyl chains of varying length, were employed to determine their efficiencies as substrates in the synthesis of MGlcDAG or DGlcDAG at saturating concentration (50 mol %) of the activator di-18:1c-PG. PC (18:1c) was used as matrix in order to avoid any substrate−product interference. Optimal rates of synthesis for MGlcDAG or DGlcDAG were obtained with a substrate chain length of 14 or 15 carbons, respectively (Figure 1). DAG analogues with ether-bound saturated chains (1,2-dialkylglycerol) could also be efficiently processed to MGlcDAG with a similar chain length dependence (data not shown). DAG with 8:0 chains could not be processed to MGlcDAG (data not shown), probably due to its high solubility in water but possibly also to a large mismatch in chain length with the 18:1-lipid matrix, which may sequester the DAG into domains.

Molecular species of DAG substrates having similar effective lengths, i.e., 18:0, 18:1c, or 18:2c chains, yielded different effects at low (5 mol %) or high (30 mol %) concentrations for the synthesis of MGlcDAG (Figure 1A). All three species gave the same rates at low (physiological) concentrations, indicating that the degree of acyl chain saturation of DAG did not influence the activity of the MGlcDAG-synthase. At high concentrations, the di-18:0-DAG was glucosylated to a similar extent as at low concentrations but significantly less than the unsaturated chain species. Due to the high melting temperature and low solubility for di-18:0-DAG (Small, 1986), only small amounts of this species can be mixed into the liquid-crystalline phase of the lipid matrix and it was therefore probably not efficiently presented to the enzyme at high concentrations. This is most likely also valid for MGlcDAG with long saturated chains, having very high chain-melting transition temperatures (>70 °C; Mannock et al., 1990a). Unsaturated DAGs have much lower T_m and higher solubilities; the effect of 18:1c-DAG at high concentration (Figure 1A) is in accordance with the previously determined concentration (Lineweaver−Burk) relationship (Karlsson et al., 1994). A similar chain length dependence for the MGlcDAG synthesis (Figure 1A) was also recorded in matrixes with small and large amounts of the nonlamellar polar lipid di-18:1c-phosphatidylethanolamine (di-18:1c-PE) (data not shown). This indicates that the substrate preferences of these enzymatic steps are not shifted by changing the phase equilibria and the spontaneous curvature (Keller et al., 1993) in the reconstituted bilayers.

The DGlcDAG synthesis rate was higher at low concentration with the 18:1c MGlcDAG species compared to the saturated 18:0 species (Figure 1B), in contradiction to the MGlcDAG synthesis. A set of MGlcDAGs with acyl chains containing methyl branches or cyclohexyl structures (Mannock et al., 1990b) were also all more efficient substrates for the DGlcDAG-synthase than the straight chain, saturated species of similar length (Figure 1B; data not shown). This was especially the case for the cyclohexyl variants. The larger bulkiness of these modified chains yields lower T_m values than for the corresponding saturated MGlcDAGs (Mannock et al., 1990b) and probably lower lamellar/nonlamellar phase transition temperatures [cf. Lewis et al. (1989)]. This indicates that increasing chain unsaturation, branching, and bulkiness (in other words, lower T_m and L_α to H_{II} transitions) for the MGlcDAG substrates stimulate the enzymatic synthesis of DGlcDAG.

When the glucolipid synthesis was studied in presence of 5 mol % of the substrate lipid DAG only, the newly synthesized fraction of MGlcDAG, constituting less than 1 mol % of the total lipids, could, in part, be further processed to DGlcDAG. The same rate of DGlcDAG synthesis could not be achieved when only <1 mol % of MGlcDAG substrate was added. The reason for this could be that (i) the consecutive syntheses of MGlcDAG and DGlcDAG are coupled, e.g., newly synthesized MGlcDAG could be "channeled" to the DGlcDAG synthase [cf. Welch & Easterby (1994)]; (ii) DAG could function as an activator of the DGlcDAG synthesis [cf. Walsh & Bell (1986)]; or (iii) changes in the physical state of the lipid matrix due to the nonbilayer properties of unsaturated DAG (de Boeck & Zidovetzki, 1989; Epand, 1985; Small, 1986) could modulate the activity of the DGlcDAG-synthase. In Figure 2 the formation of DGlcDAG was analyzed with increasing

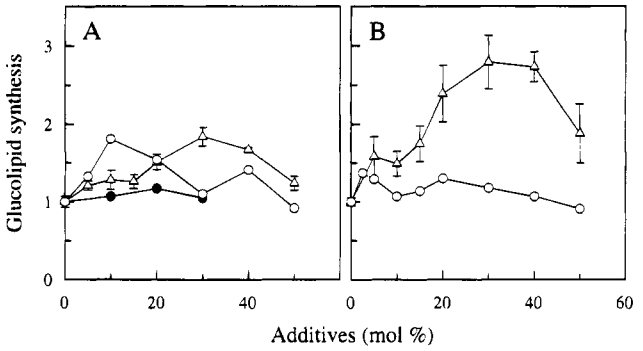


FIGURE 3: Effects of nonlamellar-forming polar lipids. Synthesis of MGlcDAG (A) or DGlcDAG (B) was measured with 5 mol % di-18:1c-DAG or 10 mol % di-18:1c-MGlcDAG substrates, respectively. Increasing amounts of polar lipids were added: di-18:1c-PE (○), or 1,2 di(2′-cyclohexylmethyl-16:0)-PC (△), in a di-18:1c-PG (40 mol %)/PC matrix; and di-18:1c-MGlcDAG (●) in a di-18:1c-PG (30 mol %)/DGlcDAG matrix. A synthesis rate of 1.0 corresponds to the absence of nonlamellar-forming additives. Mean values from three experiments. Error bars as in Figure 1, shown only for one curve.

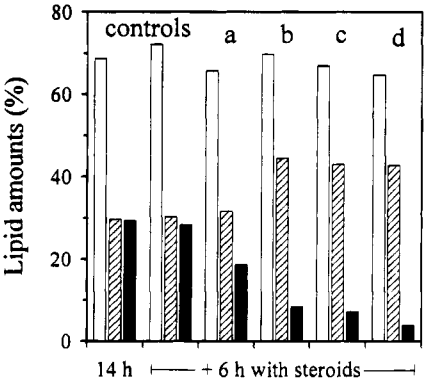


FIGURE 4: Influence of steroids on the membrane glucolipid composition. Cells were grown for 14 h at 30 °C in a medium containing 150 μM 18:1c. Portions of the culture were then diluted 50% (v/v) with media containing 60 μM (a) cholesterol, (b) epicoprostanol, (c) cholest-5-en-3-one, or (d) cholest-4-en-3-one, and incubated further for 6 h. The amounts of gluco- and phosphoglucolipids (open bars), DGlcDAG (hatched bars), and MGlcDAG (solid bars) are presented as mole percent of total lipids in the membranes. Data not previously shown from Rilfors et al. (1987).

amounts of MGlcDAG with or without 1,2-DAG. At 10 or 20 mol % of MGlcDAG the amount of glucose incorporated into DGlcDAG was increased about 2-fold in the presence of DAG. This could not be explained by further processing of newly synthesized MGlcDAG since the molar fraction of MGlcDAG increased by less than 1 mol % of the total lipids (Figure 2, legend). Most likely the increased synthesis of DGlcDAG is due to a direct activation of the DGlcDAG synthase by DAG or an indirect effect via changes in bilayer properties. This is also supported by an experiment (Figure 5) in which 1,3-DAG, which could not be processed to MGlcDAG, gave a similar increase in the DGlcDAG synthesis.

Effects of Nonlamellar-Forming Polar Lipids. In vivo the lipid bilayer of *A. laidlawii* is poised to phase equilibria close to a potential L_α to H_{II}/Q_{II} phase transition (Lindblom et al., 1986). The metabolic regulation responds strongly especially toward conditions or additives promoting this transition (Rilfors et al., 1993). Results presented in Figure 2 indicate that this may also be the case *in vitro*.

The polar lipid species di-18:1c-MGlcDAG, di-18:1c-PE and 1,2-di-(2′-cyclohexylmethyl)hexadecanoyl-PC (di-CHM-16:0-PC) all form H_{II} phases with excess water at physiological temperatures (Lindblom et al., 1986; Lewis et al., 1989, 1994). Di-18:1c-PE and di-18:1c-MGlcDAG have similarily low L_α to H_{II} transition temperatures (above), increase acyl chain order in an L_α phase (Eriksson et al., 1991; Lafleur et al., 1990), and have similar (negative) spontaneous curvature (Österberg et al., 1995). However, only the di-18:1c-PE had an activating effect, at low concentrations, on the synthesis of MGlcDAG (Figure 3A). For a bilayer of di-18:1c-PC addition of 30 mol % di-18:1c-PE changes the spontaneous curvature from 0.0104 to 0.0206 $Å^{-1}$ (or the radius from ≈96 to ≈46 Å) according to Keller et al. (1993). A similar change should be brought by di-18:1c-MGlcDAG. Therefore, curvature or chain order is probably not governing the synthesis of MGlcDAG. The stimulation by small amounts of PE is more likely an enhancement of the PG activator effect. A similar role for PE has been recorded for the activation of protein kinase C by phosphatidylserine (Newton & Koshland, 1989; Lee & Bell, 1992).

The di-CHM-16:0-PC has been shown not to be able to form a lamellar liquid-crystalline phase and has a gel to H_{II} phase transition at about 6 °C (Lewis et al., 1994). This lipid most probably has a larger H_{II} propensity than di-18:1c-PE or -MGlcDAG. The enzymatic formation of DGlcDAG was increased in a dose-dependent manner up to 3-fold with increased amounts of di-CHM-16:0-PC (Figure 3B). A less pronounced increase in the rate of synthesis was valid for the formation of MGlcDAG (Figure 3A). The influence of PE on the synthesis of DGlcDAG was minor, and much less strong than for DAG (Figure 2) or di-CHM-16:0-PC. However, all these three additives affect the amounts of PG activator needed (Figure 6).

These experiments revealed that the *in vitro* synthesis of MGlcDAG and DGlcDAG could be modulated by the presence of certain polar lipids able to form H_{II} phases. This is consistent with the organism's ability to maintain certain packing properties of the lipid bilayer *in vivo*.

Effects of Nonbilayer-Forming Hydrophobic Lipids. The shortest doubling time of *A. laidlawii* cultures is approximately 1.5 h, but with an average of 2−3 h during a culture growth cycle at 30 °C. In the present study the maximal synthesis rates of MGlcDAG and DGlcDAG were 55 and 25 nmol/mg of protein) h^{-1} *in vitro*, respectively. It can be calculated (using data from Rilfors et al., 1987, and from Nyström et al., 1992) that synthesis rates for MGlcDAG and DGlcDAG of 30 and 10 nmol per mg of cell protein per hour, respectively, are sufficient *in vivo* to maintain the glucolipid and phosphoglucolipid composition in the *A. laidlawii* membrane at average doubling times. Exposure of growing cells to new conditions can reveal differences in glucolipid composition in 10 min (Clementz et al., 1986). Can the two glucosyltransferases sense the changes in the bilayer, and are modulation of enzyme activities sufficient for the metabolic responses observed *in vivo*?

(i) Steroids. Four steroids, i.e., cholesterol, epicoprostanol, cholest-5-en-3-one, and cholest-4-en-3-one, with varying potencies to induce the L_α to H_{II} phase transition for polar lipids (Cullis & De Kruijff, 1978; Gallay & De Kruijff, 1982), were supplied by a shift-dilution technique (Figure 4; Rilfors et al., 1987). Cell growth and lipid synthesis were

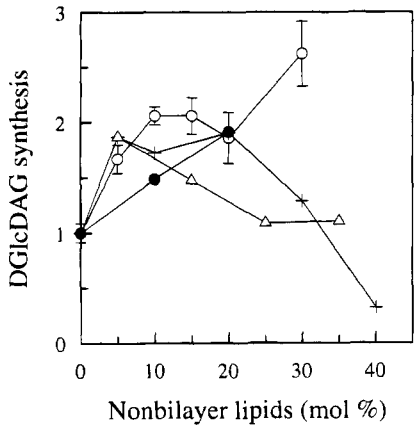


FIGURE 5: Nonbilayer lipids and DGlcDAG synthesis. The rate of DGlcDAG synthesis was measured in presence of 10 mol % di-18:1c-MGlcDAG substrate in a lipid matrix consisting of di-18:1c-PG (30 mol %) and di-18:1c-DGlcDAG, with increasing mole percent of different nonbilayer lipids: 1,3-di-18:1c-DAG (+), 5 mol % 1,3-di-18:1c-DAG plus increasing $C_{16}EO_8$ detergent (△), cholesterol (●), or cholest-4-en-3-one (○). A synthesis rate of 1.0 corresponds to no bilayer perturbants (≈7 nmol of DGlcDAG synthesized/(mg of cell protein) h^{-1}. Mean values from three experiments. Error bars as in Figure 3.

strongly retarded by the latter three of these steroids. Total membrane lipid amounts only increased 1.4 times in 6 h (doubling time 13.8 h) in the presence of cholest-4-en-3-one, compared to a 4.3-fold increase with no addition (doubling time 2.9 h). MGlcDAG decreased from 28 to 4−8 mol % while the quantity of DGlcDAG was metabolically elevated from 30 to about 43 mol % by incorporation of these steroids. However, the total glucolipid content of the membrane did not vary much (Figure 4), nor did the fractions of PG (data not shown). The metabolic changes correlate strongly to the propensities of these steroids to lower the H_{II} transition temperatures and acyl chain order in lipid bilayers [cf. Rilfors et al. (1987)]. From the new, lower rate of lipid synthesis, and hence longer doubling time after the shift, it can be calculated that the synthesis rate for DGlcDAG in relation to the rate for MGlcDAG must increase 2.6 times to account for the changes in the relative amounts of DGlcDAG brought by cholest-4-en-3-one in 6 h (according to $\ln(N_1/N_0) = k(t_1 - t_0)$; Neidhardt et al., 1990). Since the total lipid synthesis decreased and doubling times increased from 2.9 to 13.8 h (i.e., 4.8 times), the new synthesis rate for DGlcDAG is well within the the range of rates before the shift.

In 18:1c membranes *in vivo* the cholesterol content is 20−25 mol % (Rilfors et al., 1987; Wieslander & Selstam, 1987). Cholesterol and cholest-4-en-3-one had no significant effect on the MGlcDAG synthesis *in vitro* (data not shown) while more than a 2-fold increase in the synthesis of DGlcDAG could occur (Figure 5). Cholest-4-en-3-one being the more potent nonbilayer-promoting molecule (Gallay & De Kruijff, 1982) also had a stronger influence on the DGlcDAG synthesis at low (10 mol %) concentrations. Thus, these results are in good agreement with the *in vivo* studies presented above.

It can be concluded that (i) the lowered relative amount of MGlcDAG, due to the presence of different steroids, is not caused by regulation of the MGlcDAG synthase but by adjustment of the DGlcDAG synthesis; (ii) the amounts of lipids processed through the MGlcDAG-synthase is coordinated to the extent of cell growth; and (iii) the increase in DGlcDAG synthesis *in vitro* is sufficient to account for the changes in amounts recorded *in vivo*.

(ii) 1,3-Dioleoylglycerol. Under certain conditions with saturated chains *in vivo*, where MGlcDAG forms an L_α phase, 1,2-DAGs are accumulated in the membrane (Wieslander et al., 1995). DAGs can induce structural changes in lipid bilayers which are manifested as an increase in the spontaneous curvature and lower L_α to nonlamellar transition temperatures [reviewed in Goldberg et al. (1994)]. The lipid substrate 1,2-di-18:1c-DAG appeared to stimulate the synthesis of DGlcDAG (Figure 2). 1,3-di-18:1c-DAG could not be processed to MGlcDAG (data not shown). Since it has similar effects on phase equilibria of bilayers as the corresponding 1,2-DAG (van Gorkom et al., 1992), it would be anticipated that it would promote the DGlcDAG biosynthesis in an analogous manner. An almost 2-fold increase in the rate of synthesis was achieved with 5 mol % of 1,3-DAG, and higher concentrations did not further elevate the DGlcDAG synthesis (Figure 5). However, unsaturated DAGs induce the formation of nonlamellar phases in lipid bilayers at fairly low concentrations (Das & Rand, 1986; Epand, 1985), and the decrease in synthesis rate above 20 mol % DAG (Figure 5) coincides with the appearance of a two-phase region ($L_\alpha + H_{II}$) for DAG in PC (Das & Rand, 1986; de Boeck & Zidovetzki, 1989).

If the enhancement of DGlcDAG synthesis by DAG is caused by the increased propensity of the membrane to form nonlamellar phases and not by specific interactions, then the origin of the perturbing molecules present in the bilayer should be of no importance. Thus, incorporation of detergents having opposite effects on the phase equilibra should counteract the stimulation of the DGlcDAG biosynthesis caused by the nonlamellar-promoting molecules. Elevated amounts of the detergent $C_{16}EO_8$ was able to counterbalance the activation caused by 1,3-di-18:1c-DAG (Figure 5). No inhibition was found when the detergent was added to a matrix consisting of lamellar lipids only. This and the similar $C_{12}E_8$ detergent were found to induce the formation of normal hexagonal (H_I) phases in PC bilayers (Beyer, 1983; Thurmond et al., 1994b). These results indicate that the glucolipid syntheses are only regulated in response to packing properties within a range of curvature which is relevant for the *in vivo* conditions. In 18:1c-enriched membranes, 10−30 mol % nonbilayer-preferring lipids are always present (Wieslander et al., 1995).

Our findings suggest that the enzymatic activity of the DGlcDAG synthase is correlated with the potential abilities of the lipid bilayers to form type II nonlamellar phases or with an increased (negative) spontaneous curvature. The response caused by DAG also indicates that this molecule can have a functional role as a packing agent under certain circumstances *in vivo*, which has been suggested recently (Wieslander et al., 1995).

Enhanced Activation by Phosphatidylglycerol in the Presence of Nonbilayer Perturbants. The physiological concentration of PG in the membranes of *A. laidlawii* can vary between 10 and 30 mol % depending on growth conditions (Wieslander et al., 1995) but experience no rapid changes as can the glucolipids. In order to elucidate how bilayer-perturbing molecules affect the activating potency of PG, 1,3-di-18:1c-DAG or cholest-4-en-3-one was incubated with increasing concentrations of PG in a DGlcDAG lipid matrix

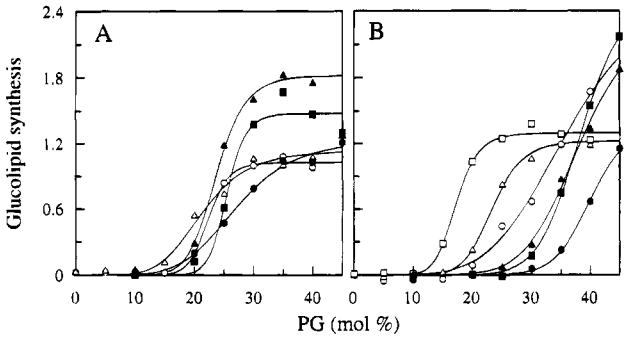


FIGURE 6: Potentiation of phosphatidylglycerol activator by different nonbilayer lipids. The synthesis of MGlcDAG (A) or DGlcDAG (B) was assayed in the presence of 5 mol % di-18:1c–DAG or 10 mol % di-18:1c-MGlcDAG substrates, respectively, plus 0%–45% di-18:1c-PG in a di-18:1c-DGlcDAG matrix (○), with 15 mol % of cholest-4-en-3-one (△) or 1,3-DAG (□); and in a di-18:1c-PC matrix (●), with 15 mol % of di-18:1c-PE (▲), or 1,2 di-(2′-cyclohexylmethyl-16:0)-PC (■). The rates (mean values from three experiments) were normalized to values obtained in presence of 30 mol % PG in a DGlcDAG matrix with no perturbants. The curves are obtained from simple fits of the nonlinear Hill equation, $v = V_{max}/(1 + (K/[L]^n)$ [cf. Karlsson et al. (1994)].

(Figure 6). The activation curves for the DGlcDAG synthesis were shifted substantially toward lower PG concentrations. Similar, but less pronounced features for the DGlcDAG synthesis were also observed for two different H_{II}-forming phospholipids, di-CHM-16:0-PC and di-18:1c-PE in a di-18:1c-PC matrix. Both lipids lowered the PG concentrations needed for activation of the DGlcDAG synthesis (Figure 6B, solid symbols). Hence, nonlamellar-promoting additives can increase the synthesis rate for DGlcDAG in both DGlcDAG and PC lipid matrixes, but the activation is dependent upon PG. 1,3-DAG had the largest influence on the activation curve by lowering the fraction of PG required for half-maximal activation (K in equation of Figure 6 legend) by approximately 20 mol %. The amount of PG needed for activation of MGlcDAG synthesis (Figure 6A) was only slightly influenced by the additives. At high concentrations of PG (30–45 mol %) both the zwitterionic lipids di-18:1c-PE and the di-CHM-16:0-PC increased the maximum rate of synthesis for MGlcDAG, while at low, physiological PG concentrations the effects of these additives were much less pronounced. The increase may be caused by an increased surface charge density due to these two phospholipids.

It can be concluded that at physiological concentrations of PG different nonlamellar-promoting perturbants can strongly enhance the synthesis of DGlcDAG in relation to their nonbilayer propensities. This was valid in two different bilayer matrixes and involved increased activating efficiences of PG in the presence of the perturbants.

CONCLUSION

It is shown here that several basic features of the homeostasis mechanism in *A. laidlawii*, maintaining similar phase equilibria, spontaneous curvature, and surface charge density for the membrane lipid bilayer, are dependent upon the physical properties in the bilayer and sensed by the two consecutively acting glucosyltransferases synthesizing the major lipids MGlcDAG and DGlcDAG.

Activation by Phosphatidylglycerol. Fairly high, but physiological, amounts of an essential activator lipid are needed for both of the glucosyltransferases. The demand for a liquid-crystalline PG by the DGlcDAG enzyme is specific; short-chain or gel-phase variants of PG are much less efficient (Table 1). Potent bilayer-modulating molecules cannot release the requirement for the PG activator (Figure 6). The two biosynthetic pathways from the precursor PA to the PG activator and the glucolipids, respectively (see introduction), are mutually connected to each other in several aspects. First, the synthesis of MGlcDAG is activated jointly by the anionic PG and phosphoglucolipids (Karlsson et al., 1994). The purpose most likely is to maintain a constant surface charge density (Christiansson et al., 1985). Second, the acyl chain preferences of the MGlcDAG synthase *in vitro* yield more MGlcDAG with DAG substrates containing shorter, saturated chains (Figure 1). This is also observed *in vivo* (Christiansson & Wieslander, 1980), as is the correspondingly decreased metabolic flow into the PG pathway with decreasing chain length and decreased chain unsaturation, respectively (Wieslander et al., 1995). Third, the DGlcDAG synthase is activated in a cooperative fashion *in vitro* by the presence of PG, but the amounts needed vary depending on the packing conditions in the bilayer (Figure 6). *In vivo* there is a close correspondence between the amounts of PG and DGlcDAG as a function of acyl chain length and unsaturation, where these two lipids vary between approximately 5 and 27 mol % and 5 and 50 mol %, respectively (Wieslander et al., 1995). Most important, PG amounts are not metabolically adjusted in a rapid manner as is DGlcDAG (Wieslander et al., 1986; Rilfors et al., 1987), indicating that PG is not regulated by lipid packing parameters.

Regulation of MGlcDAG and DGlcDAG Amounts. The rate of synthesis of MGlcDAG seems to be mainly regulated by the amounts of activator lipid, and by substrate concentrations and chain preferences, respectively (Figures 1 and 6). No consequent, and only minor, variations in activities as a function of nonbilayer lipids, curvature, or chain order were observed. Hence, the strong increase in MGlcDAG amounts with shorter acyl chains *in vivo* (Thurmond et al., 1994a; Wieslander et al., 1995), must be governed by the increased synthesis with short-chain DAG substrates (Figure 1A).

The activity of the DGlcDAG-synthase was clearly more responsive to packing conditions in addition to substrate properties. First, nonlamellar-promoting molecules stimulated the synthesis of DGlcDAG *in vitro* in a dose-dependent manner (Figures 3 and 5). Second, there was a correspondence between the nonbilayer propensities of these additives and the responses. Polar lipids were active but less efficient than the more hydrophobic ones (Figures 3, 5, and 6). Third, the reduction in activities brought by an H_I-promoting (micelle-forming) detergent was only achieved in the presence of 1,3-DAG (Figure 5). This and the second point above suggest that the enzyme is only responsive within a certain, restricted window of curvature. Fourth, at low concentrations, long unsaturated or branched MGlcDAG substrates yielded higher activities than the corresponding saturated ones, in accordance with the increase in DGlcDAG amounts occurring *in vivo*.

Comparison with Other Systems. There are striking similarities in the importance of the lipid surface charge density, essential activator lipids, and modulation by nonbilayer lipids or the spontaneous curvature for these two membrane-bound glucosyltransferases and certain other

enzymes. These include both peripheral (Kinnunen et al., 1994) and certain integral proteins, like protein kinase C (Epand et al., 1991; Goldberg et al., 1994; Mosior & Epand, 1993; Slater et al., 1994), phospholipase A_2 (Sen et al., 1991; Zidovetzki et al., 1992), phosphocholine cytidylyltransferase (Cornell, 1991a,b), mannosyltransferase (Jensen & Schutzbach, 1988), Ca^{2+}-ATPase (Cheng & Hui, 1986), rhodopsin (Gibson & Brown, 1993), and the insulin receptor (McCallum & Epand, 1995).

Model for Regulation. The regulation of the MGlcDAG synthesis probably depends upon an electrostatic approach of the membrane-anchored enzyme to a membrane surface with a proper lipid charge density or potential and which contains the DAG substrate.

The regulation of the DGlcDAG synthesis may depend upon one or more of the following: (i) The enzyme is directly activated conformationally by binding of PG, in combination with the packing strain given by the spontaneous curvature. The structure of the analogous monogalactosyl-DAG-synthase from chloroplast envelopes is affected by PG (Maréchal et al., 1995). (ii) The access of the enzyme to substrate or activator lipids, i.e., structural conformations of the polar heads or domain formation of these lipids, depends upon the packing properties in the bilayer. (iii) The enzyme is a di- or oligomer, and the lateral aggregation of the subunits to an active complex is dependent upon the unique properties of PG, the type of matrix, and the extent of the spontaneous curvature. Any one of these theoretical alternatives is amenable to experimental testing in proper model systems.

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