Transmembrane Electrical Potential Affects the Lipid Composition of *Acholeplasma laidlawii*[†]

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ABSTRACT: In membranes of *Acholeplasma laidlawii*, lipid composition is regulated as a function of several stimuli affecting the volume and length of the hydrocarbon chains and the hydrocarbon–water interfacial area. This regulation is vizualized as changes in the relative amounts of the major polar lipids monoglucosyl diglyceride and diglucosyl diglyceride. These lipids form reversed hexagonal and lamellar phases with water, respectively. However, mixtures of the two lipids, in the molar proportions found in the *A. laidlawii* membrane, form a lamellar phase. By adjustment of the glycolipid ratio as a response to environmental stimuli, a certain stability of the lamellar membrane is maintained. In growing cells with oleoyl membrane lipids, a transmembrane electrical potential of approximately −50 mV (inside negative), but no transmembrane pH difference, was found. Addition of the K^+ ionophore valinomycin caused a rapid and dose-dependent hyperpolarization remaining for at least 7 h. Simultaneously, a rapid and lasting metabolic *decrease* in the ratio monoglucosyl diglyceride/diglucosyl diglyceride occurred. The *increase* in potential and the *decrease* in the lipid ratio were both reversed in a dose-dependent manner by extracellular KCl. Likewise, the lipophilic cation tetraphenylphosphonium caused a dose-dependent *decrease* in membrane potential and an *increase* in the monoglucosyl diglyceride/diglucosyl diglyceride ratio, respectively. The ionophores monensin and particularly nigericin had similar but less pronounced effects on the potential and lipid ratios as valinomycin. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone had no effect on cell growth, membrane potential, or lipid regulation at 10 μM. These dissimilar structures and the low concentrations used make a direct disturbance of drug molecules on lipid packing in membranes less likely. The coherent variation of lipid composition with the membrane potential supports an effect of the electrical potential on lipid packing and lamellar stability similar to what can be observed in model lipid systems.

The main function of biological membranes, in addition to physical containment, is to act as an insulating barrier with selective permeability properties and as a matrix for enzyme systems. A lamellar phase of lipids with embedded proteins is considered to be the structural base of the membrane. In the membrane of the cell wall less procaryote *Acholeplasma laidlawii*, certain packing properties are actively maintained by a mechanism that regulates polar lipid composition and is sensitive to endogenous as well as exogenous stimuli. By adjustment of the relative amounts of the monoglucosyl diglyceride (MGDG)[1] and diglucosyl diglyceride (DGDG), forming reversed hexagonal (H_{II}) and lamellar phases, respectively, similar phase behavior in the lamellar membrane lipid mixture is maintained irrespectively of the actual acyl chain composition, steroid supplement, or growth temperature in this fatty acid auxotrophic organism (Wieslander et al., 1980; Rilfors et al., 1984; Rilfors, 1985). Furthermore, a constant lipid surface potential is maintained (Christiansson et al., 1985).

In most cells and organelles, membranes are involved in the energy metabolism by separating compartments with different proton and monovalent cation concentrations. The maintenance of these concentration differences is crucial for the storage and utilization of chemiosmotic energy. The insulating properties of lamellar membranes are to a large extent determined by the chemical structure and physical state of their lipids (Boheim et al., 1980; Hopper et al., 1970). The water channels in nonlamellar aggregates lack the insulating properties of lamellar membranes. Hence, a coupling between energy state and membrane structure is likely to occur in living systems. In lipid model systems, application of transmembrane electrical potential strongly affects both the permeability and structural properties of lipid bilayers [e.g., see Shchipunov & Drachev (1982)]. The influence on membrane molecular organization has also been observed in vivo (Amar et al., 1978; Bevers et al., 1978; Donohue-Rolfe & Schaechter, 1980; Langley & Kennedy, 1979).

A. laidlawii grows at basic pH, ferments glucose to lactate, and obtains ATP from substrate-level phosphorylation (Pollack et al., 1983). By the use of ionophores known to influence the electrochemical potential across membranes, we show here that in *A. laidlawii* a connection between the transmembrane electrical potential and the regulation of polar lipid composition exists. This regulation, expressed as the relative amounts of MGDG over DGDG, is intimately connected with the lamellar

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[1] Abbreviations: FA, fatty acyl chains; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyloxazolidine-2,4-dione; TPP^+, tetraphenylphosphonium ion; MGDG, monoglucosyl diglyceride; DGDG, diglucosyl diglyceride; $\Delta\psi$, transmembrane electrical potential; ΔpH, $pH_{in} - pH_{out}$; $\Delta\mu_{H^+}$, $\Delta\psi - (2.3RT/F)\Delta pH$; $\Delta\mu_{K^+}$, $\Delta\psi - (2.3RT/F) \log ([K^+]_{in}/[K^+]_{out})$; Tris, tris(hydroxymethyl)aminomethane.

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phase stability of *A. laidlawii* membrane lipids.

MATERIALS AND METHODS

Strain and Growth Conditions. Acholeplasma laidlawii strain B(ju) was used. It was adapted to growth in a lipid-depleted bovine serum albumin-tryptose medium (Christiansson & Wieslander, 1980) supplemented with 150 μM oleic acid. The membrane lipids were radioactively labeled by the addition of 10 μCi/L [^{14}C]oleic acid. In control experiments, the medium was also supplemented with [^{3}H]acetate to monitor the synthesis of endogenous saturated fatty acyl chains (Christiansson & Wieslander, 1980). Growth was analyzed by measurement of the cell culture turbidity at 540 nm and by estimation of membrane amounts by lipid quantification (see below). All the experiments were started at a turbidity of approximately 0.3, corresponding to 0.1 mg of cell protein/mL.

Lipid Analysis. The cells were harvested by centrifugation at 32000*g* for 8 min at 5 °C and washed once in β buffer (Christiansson & Wieslander, 1978). Membranes were prepared by osmotic lysis and washed twice in β buffer diluted 1/20 (v/v). Lipids were extracted, purified, separated by thin-layer chromatography, and quantified by liquid scintillation counting as described (Christiansson & Wieslander, 1978). Gas–liquid chromatography was used to analyze the total fatty acyl chain composition of the membranes (Christiansson & Wieslander, 1980).

Shift Experiments with Different Drugs. Cells were grown at 30 °C to a turbidity of 0.3 (approximately 14 h). At this time, the culture was divided, and each part was supplemented with the appropriate drug. The volume of the ethanolic solution of the added drugs never exceeded 1% of the total volume. Control cultures were supplemented with the corresponding amount of ethanol. Growth was allowed to proceed for 6–9 h at 30 °C. Measurements of culture turbidity in the presence of the various drugs were used for titration of appropriate concentrations.

Leakage of Intracellular Material. The determination of intracellular water space is dependent upon an intact membrane. To examine if this criterium was fulfilled during drug treatment and subsequent methodological steps, the cells were tested for leakage of [^{3}H]uridine-labeled cellular material. *A. laidlawii* was grown as described above in medium supplemented with 10 μCi/L [^{14}C]oleic acid. In addition, the medium was supplemented with 200 μCi/L [^{3}H]uridine. The cells were washed once and resuspended to 0.25 mg of cell protein/mL in fresh medium without any radioactive labels. The ratio of ^{3}H dpm to the nanomoles of fatty acyl chain (FA) in this suspension was set to 100%. After treatment with gramicidin D (10 nM) and subsequent centrifugation through silicone oil (see below), the retained intracellular material was 68–89%. This indicates an extensive and unpredictable loss of intracellular material when cells are exposed to gramicidin. With all the other drugs tested, including controls, the retained material was 96% ± 1.5%.

Determination of Intracellular Water Space. As *A. laidlawii* lacks a rigid cell wall, the intracellular volume might change in response to changes in the osmotic pressure. Such changes could be induced by the free flow of ions across the membrane caused by the addition of ionophores. Determination of intracellular water space was made as described by Rottenberg (1979) with ^{3}H$_2$O as a permeable probe and [^{3}H]inulin as a nonpermeable probe. The intracellular water space was correlated to the amount of membrane lipids. Determination of lipid acyl chains proved to be a method with greater precision and less fluctuations between experiments than determination of total cell protein. For conversion of volume per micromole of FA to volume per milligram of cell protein, a factor of 1 μmol of FA per 3 mg of cell protein was established. Protein was determined by the method of Hartree (1972) with bovine serum albumin (Cohn V) as standard. The results are shown in Table I.

Table I: Intra- and Extracellular Water Space in *A. laidlawii* Used for Calculation of Intracellular Concentrations[a]

	intracellular water space		
drug treatment	μL/μmol of FA[b]	μL/mg of cell protein	extracellular water space (%)[c]
control (1% EtOH)	19.2 ± 2.8	6.0 ± 0.9	4.9 ± 2.3
valinomycin (1 μM)	18.8 ± 3.5	5.9 ± 1.1	4.3 ± 3.4
TPP (250 μM)	18.5 ± 4.6	5.8 ± 1.4	6.7 ± 2.8
nigericin (25 nM)	22.5 ± 4.1	7.0 ± 1.3	6.5 ± 2.7
monensin (1 μM)	21.9 ± 4.9	6.8 ± 1.5	5.3 ± 1.9
CCCP (10 μM)	18.8 ± 4.3	5.9 ± 1.3	5.6 ± 2.2

[a] Values are presented with 90% confidence limits. [b] FA, fatty acyl chains in membrane lipids (^{14}C labeled). [c] Expressed as the percent extracellular water of total water space in the pellet.

Measurements of $\Delta\psi$ and ΔpH. The electrical potential ($\Delta\psi$) across the membrane was determined as introduced by Liberman et al. (1969), by measuring the accumulation of a lipophilic cation. In our work, we used [^{3}H]tetraphenylphosphonium ([^{3}H]TPP$^+$) and determined probe distribution by centrifugation of cells. Suspensions of growing cells (in growth medium) were incubated with [^{3}H]TPP$^+$ (2 μCi/mL) (24 Ci/mmol). After 5 min, the suspension was supplemented with the appropriate drug and incubated for another 15 min or 7 h at room temperature. Control samples were incubated with a corresponding amount of ethanol. After the incubation, 400-μL samples were transferred to prechilled (5 °C) Eppendorf tubes containing 1 mL of silicone oil [55% Wacker AR200 + 45% Wacker AR20 (w/w)]. The tubes were centrifuged immediately in a Beckman microfuge B for 3 min a 5 °C. After centrifugation, a sample was taken from the supernatant. Most of the silicone oil was removed through aspiration, and the tip of the tube, together with the cell pellet, was cut off directly into a scintillation vial. The supernatant samples were counted in a two-channel liquid scintillation counter in a cocktail consisting of 14 mL of toluene containing 4 g/L Omnifluor (New England Nuclear) and 6% (v/v) Biosolv (Beckman). Quench corrections were made by the external standard channels ratio method. The pellet samples were incubated with 1.5 mL of Protosol (New England Nuclear) plus 50 μL of H_2O for 2 h at 55 °C. The pellet samples were counted as above except for the scintillation cocktail, which contained 8 g of Omnifluor/L of toluene and no Biosolv. By this procedure, 85–95% of the original cells were pelleted and contained 95–98% of their original intracellular material as measured by leakage of [^{3}H]uridine (see above). The concentration of TPP$^+$ was never more than 0.1 μM, except for the experiments with high concentrations (>50 μM), and did not cause any significant depolarization.

The transmembrane pH difference, ΔpH, was determined by measuring the accumulation of the weak organic acid 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione ([^{14}C]DMO) (Waddell & Butler, 1959) after centrifugation of cells. Except for the exchange of [^{3}H]TPP$^+$ for [^{14}C]DMO, and the substitution of [^{3}H]oleic acid for [^{14}C]oleic acid in the growth medium, the procedures were the same as for the determination of $\Delta\psi$. The external pH was measured with a combined glass–reference electrode (Radiometer) in the cell suspension. Corrections have not been made for probe binding to the cells. Binding of probe molecules to growth medium components was less than 5%. $\Delta\psi$ was also determined in centrifuged cells suspended in a Tris-HCl buffer containing 50 mM Tris, 130 mM NaCl, 2 mM $MgCl_2$, 5 mM $CaCl_2$, and 1.2 mM Na_2HPO_4 adjusted to pH 7.4 with HCl + 20 mM glucose at different pHs between 6.4 and 8.4 and KCl concentrations between 0 and 10 mM. The $\Delta\psi$ of cells suspended in buffer without added drugs was the same as for cells in growth medium. However, no response to drug treatment could be detected in cells suspended in buffer. Calculation of $\Delta\psi$ from TPP^+ distribution and calculation of ΔpH from DMO distribution were essentially as described by Rottenberg (1979). Corrections were made for the actual amount of pelleted cells as described above and for extracellular water space in the pellet.

Measurement of Intracellular K^+ Concentrations. The internal concentration of potassium was determined by the use of ^{42}K. Cells were grown and supplemented with drugs as described in the shift experiment above, except for the presence of 3.3 mCi/mL ^{42}K (specific activity 0.99 mCi/mmol) in the medium during growth. At different times after the addition of drugs, samples were taken, centrifuged, and counted as described for $[^3H]TPP^+$ above. Corrections were made for radioactive decay during the experiment. Potassium concentration in the growth medium was determined by atomic absorption spectrometry.

Materials. Valinomycin, monensin, and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma; gramicidin D was from Serva; nigericin was from Calbiochem. The lipophilic cation TPP^+ was obtained from Merck as tetraphenylphosphonium bromide. All radioactive compounds were from Amersham. The silicone oils AR20 and AR200 were from Wacker and were purified from macroscopic debris by centrifugation at 10000*g* for 20 min. In the early phase of this investigation, radioactive TPP^+ was obtained from Dr. H. R. Kaback.

RESULTS

Growth of A. laidlawii in the Presence of Ionophores. Several inhibitors known to interfere with the electrochemical potential were tested for their ability to reduce growth in *A. laidlawii* at different concentrations. These inhibitors were the following: valinomycin, a potassium ionophore; nigericin, a potassium–proton antiport ionophore; monensin, a sodium–proton antiport ionophore; gramicidin, a hydrogen quasi-ionophore; and the lipophilic cation TPP^+. The growth rate of *A. laidlawii* was decreased in a concentration-dependent manner by each of the inhibitors as analyzed by turbidity measurements. The addition of 20 mM KCl helped the cells to maintain growth more efficiently in the presence of valinomycin, indicating that a change in the potassium gradient across the cell membrane was involved in the decreased growth rate. The effect on lipid amounts at the concentrations finally chosen for the lipid regulation experiments is shown in Table II. Swelling of the cells was only observed with gramicidin. Very small morphological changes were displayed with the other ionophores. The uncoupler CCCP was also tested. CCCP did not affect cell growth at a concentration of 10 μM.

Regulation of Membrane Lipid Composition. Most likely, any disturbing effects on the molecular packing in *A. laidlawii* membranes, caused by the ionophores themselves or by changes in the membrane potential, will be sensed by the cells and compensated for. For lipids which contain oleoyl chains only, compensatory changes in lipid composition occurring as a response to changes in the lipid and protein packing in the membrane must be accomplished at the level of polar head-group composition. The extent of endogenous saturated fatty acid synthesis in cells grown with only oleic acid was monitored by $[^3H]$acetate incorporation (Christiansson & Wieslander, 1980). In none of the experiments was there any significant incorporation of acetate into lipids. This low endogenous fatty acid synthesis was also confirmed by gas–liquid chromatography, and the membrane lipids thus contained at least 95% oleoyl chains. Differences in polar head-group composition and structure for oleoyl lipids have a profound impact on the phase equilibria in model systems containing mixtures of lipids from *A. laidlawii* (Rilfors et al., 1984; Wieslander et al., 1981). Particularly the mono- and diglucosyl diglyceride ratio is a sensitive monitor of packing properties. This means that any disturbance in the optimal lipid packing will be reflected as a change in the membrane lipid composition. This change aims to counteract the disturbance (Rilfors et al., 1984).

Table II: Lipid Amounts and Glycolipid Ratio after Drug Treatment[a]

t (h)	drug treatment	amount of glycolipid[b] (mmol/mL)	MGDG/DGDG[c]
0	control	7.1	0.94
6	control (1% EtOH)	15.8	0.97
	valinomycin (7.5 nM)	10.9	0.39
	valinomycin (7.5 nM) + KCl (20 mM)	13.8	0.64
	gramicidin (3 nM)	10.3	0.80
	TPP^+ (500 μM)	12.1	1.25
	monensin (100 nM)	12.4	0.54
	nigericin (2 nM)	11.7	0.50
	CCCP (10 μM)	16.0	0.92

[a] At a turbidity of ~0.3 at 540 nm, the culture was divided and treated with the drugs indicated (*t* = 0). [b] The amount of glycolipid is expressed as the sum of monoglucosyl diglycerides and diglucosyl diglycerides per milliliter of cell culture. The glycolipids/phospholipids ratio was constant (~1.8). [c] Molar ratio.

Effect of Valinomycin. Of the ionophores examined, valinomycin was chosen as the main subject for further studies since the use of this drug makes it possible to specifically manipulate the transmembrane electrical potential (Pressman, 1976; Reed, 1979). Upon addition of valinomycin to cells in the early logarithmic phase of growth, a rapid and substantial decrease in the monoglucosyl diglyceride/diglucosyl diglyceride ratio was obtained (Figure 1A). The change could be detected in less than 10 min after addition (Figure 1B) and was manifested at a concentration as low as 3 nM. Assuming complete uptake of valinomycin in the membrane, 3 nM corresponds to a ratio of fatty acyl chains to valinomycin of ca. 4000 to 1. The response in the glycolipid ratio was not accompanied by any significant change in the amount of charged lipid (data not shown), indicating that the membrane lipid surface potential was not changed [cf. Christiansson et al. (1985)].

Valinomycin exerts its action by being able to diffuse through the membrane, carrying a potassium ion caged inside the molecule and shielding it from the hydrophobic interior of the membrane (Pressman, 1976). When potassium was added externally, to a concentration of 20 mM along with valinomycin, the effect observed on the MGDG/DGDG ratio when valinomycin was added alone was partially counteracted (Figure 1A). Furthermore, an increase in the external potassium concentration, after the effect of added valinomycin was expressed, rapidly reversed the decrease in the MGDG/DGDG ratio (Figure 1B).

Transmembrane Electrical Potential. The diffusional reaction of valinomycin is driven by both the transmembrane concentration gradient and the electrical potential; i.e., the electrochemical activity of K^+ on both sides of the membrane tends to equalize (Pressman, 1976). The K^+ concentration

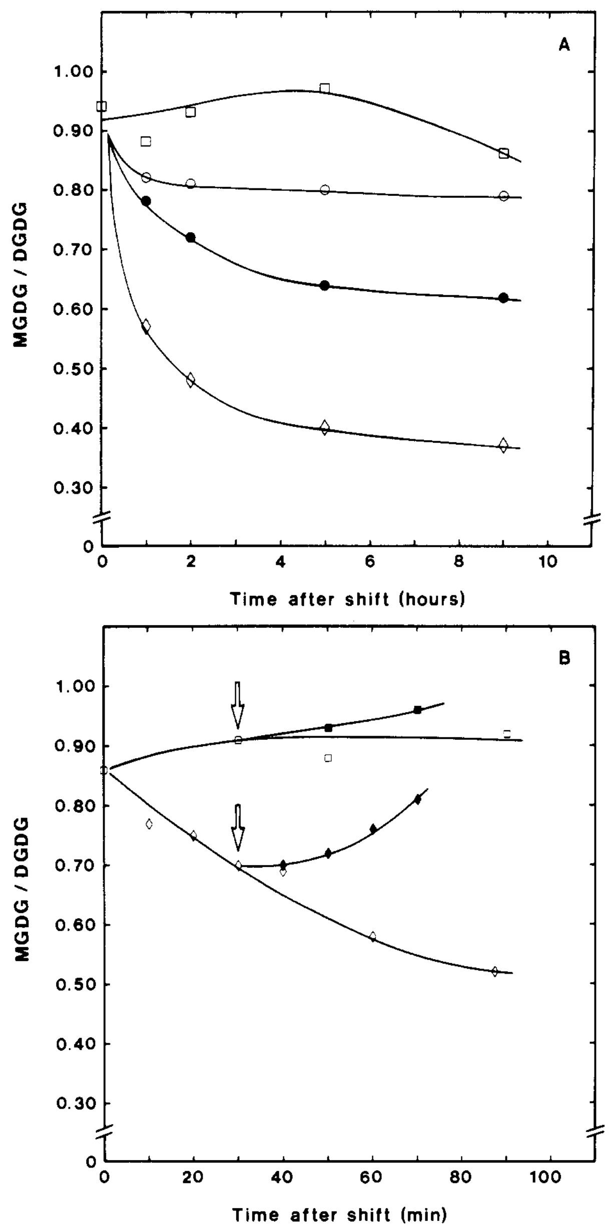

FIGURE 1: Effect of valinomycin and gramicidin on the regulation of the polar lipids monoglucosyl diglyceride and diglucosyl diglyceride. A culture of *A. laidlawii* in early logarithmic phase was divided and supplemented with ionophores at time zero. The growth medium contained 150 μM [^{14}C]oleic acid. Membrane lipid composition was determined by analysis of radioactive lipids. The glycolipid composition is expressed as the molar ratio of monoglucosyl diglyceride to diglucosyl diglyceride. (A) Effect of the ionophores: (□) control; (○) gramicidin (3 nM); (◇) valinomycin (7.5 nM); (●) valinomycin (7.5 nM) + KCl (20 mM). (B) The fast response in the glycolipid ratio upon addition of valinomycin and the reversal of the effect with KCl. Arrows indicate the time at which KCl was added to a final concentration of 50 mM. (◆) Valinomycin (7.5 nM) + KCl (50 mM); (■) control + KCl (50 mM). Other symbols as in (A).

in the *A. laidlawii* cell was determined to be 190 mM as compared to a concentration of 3.3 mM in the growth medium. Addition of valinomycin can thus be expected to lead to an outward flow of K$^+$ until this flow is counteracted by an increased electrical potential. The potassium outflow as well as the increase in potential was confirmed in *A. laidlawii.*

The electrical potential across the membrane of untreated *A. laidlawii* cells, as measured by the distribution of [^{3}H]TPP$^+$ after centrifugation of the cells through silicone oil, had a value of ca. −50 mV (Figure 2, arrow). Upon addition of valinomycin to the cells, the membrane was hyperpolarized (Figure 2). Although the extent of hyperpolarization was small, its magnitude was dose-dependent (Figure 2). At the same time, the intracellular concentration of potassium decreased from 190 to 45 mM at 1 μM valinomycin. The effect of valinomycin on $\Delta\psi$ could be detected at a concentration as low as 1 valinomycin molecule per 2000 membrane fatty acyl chains (Figure 2). This lower limit is in accordance with the concentration used in the experiments on lipid regulation. Furthermore, the cells remained hyperpolarized even after 7-h incubation at 30 °C in the presence of valinomycin (Figure 2).

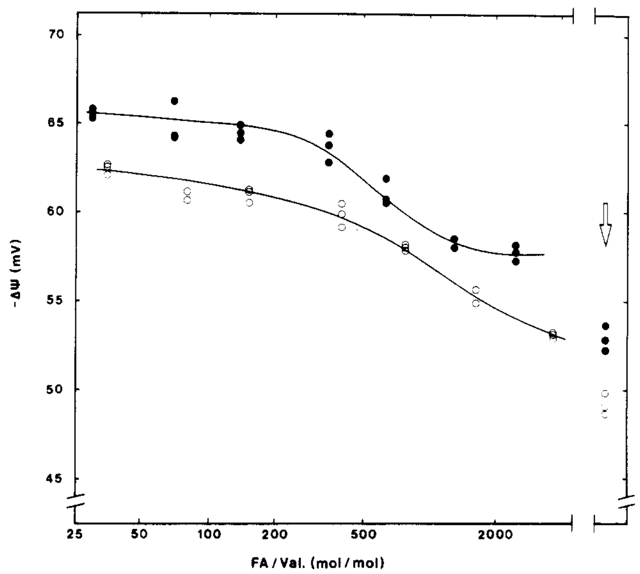

FIGURE 2: Dose-dependent response of the transmembrane electrical potential, $\Delta\psi$, upon addition of valinomycin. The potential-dependent distribution of [^{3}H]TPP$^+$ (0.1 μM) was determined after centrifugation of the cells through silicone oil. The arrow denotes $\Delta\psi$ in the absence of added valinomycin. Measurements were made at 15 min (●) and 7 h (○) after the addition of tetraphenylphosphonium bromide. FA/Val, ratio of fatty acyl chains to valinomycin in membranes, assuming a complete uptake of valinomycin into membranes.

The ability of different external K$^+$ concentrations to counteract the effect induced by valinomycin on the MGDG/DGDG ratio was examined more carefully at a valinomycin concentration of 7.5 nM (Figure 3A). When the K$^+$ concentration increased, the valinomycin-induced effect was gradually diminished, and the MGDG/DGDG ratio approached the control level. The effect of K$^+$ concentration was much less pronounced in control cells (Figure 3A). Furthermore, as the K$^+$ concentration increased, the hyperpolarizing effect of valinomycin was gradually diminished (Figure 3B). In the presence of valinomycin, a change in external potassium concentration will alter the balance between the outward driving force of the concentration gradient and the inward driving force of the electrical potential. A valinomycin concentration of 1 μM was used when $\Delta\psi$ was measured in order to get larger and more reproducible $\Delta\psi$ readings. However, the hyperpolarization was also present at 7.5 nM valinomycin, although smaller (Figure 2). These experiments thus support a direct or indirect correlation between the electrical potential and the regulation of the MGDG/DGDG ratio.

Transmembrane Proton Gradient. It has been proposed that changes in ΔpH might affect the conformation, and thus probably the packing properties, of the membrane proteins (LeGrimellec et al., 1982). Under the conditions of growth employed in our experiments, i.e., in a Tris-buffered growth medium with an initial pH of 8.4 and with only a small decrease in the pH of the medium (0.1–0.3 pH unit) during

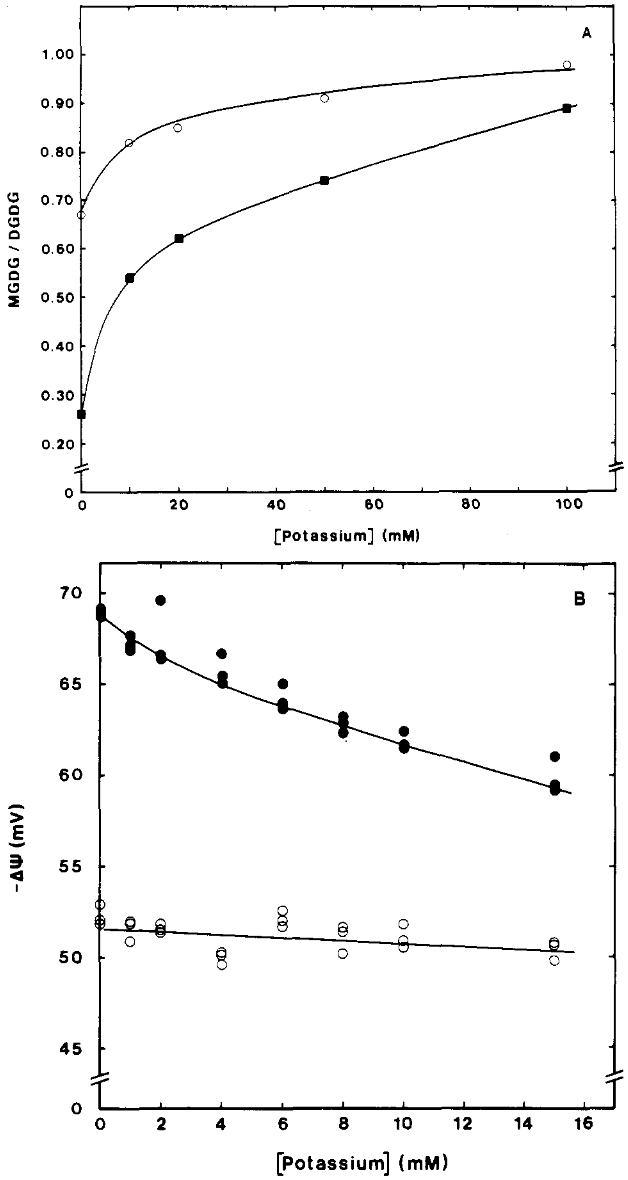

FIGURE 3: Reduction of valinomycin-induced effects by the presence of increasing concentrations of potassium. Cells were grown until early log phase. At this time, small volumes of concentrated solutions of KCl were added to increase the K^+ concentrations. Growth was allowed to continue in the presence or absence of valinomycin. $\Delta\psi$ was determined after 15 min, and the lipid composition was analyzed after 8-h growth. As the effect on the glycolipid ratio was most pronounced at potassium concentrations below 20 mM, $\Delta\psi$ was only determined in the interval 0–15 mM. Effect on (A) lipid regulation and (B) $\Delta\psi$: (○) control; (■) valinomycin (7.5 nM); (●) valinomycin (1 μM).

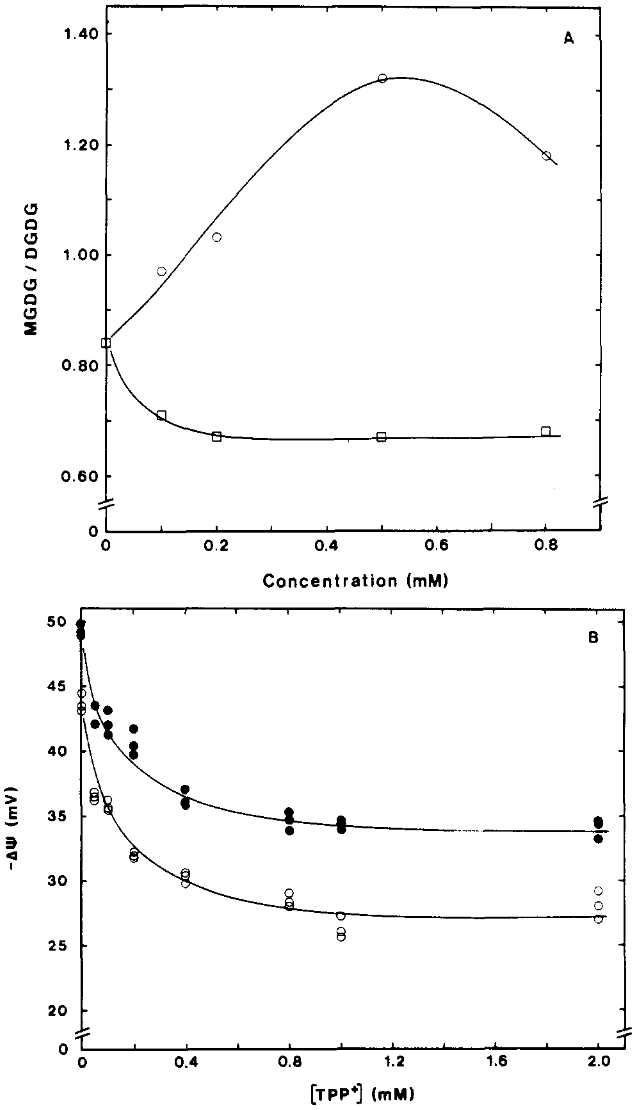

FIGURE 4: Effect of high concentrations of TPP^+. Growth conditions as in Figure 3 (without added KCl) except that TPP^+ was added instead of valinomycin. $\Delta\psi$ was determined as in Figure 2. (A) Lipid regulation: (□) control, tetraphenylphosphonium bromide was substituted for NaCl; (○) tetraphenylphosphonium bromide. (B) $\Delta\psi$: measurements were made at 15 min (●) and 7 h (○) after the addition of tetraphenylphosphonium bromide.

growth, ΔpH only showed small fluctuations (less than 0.1 pH unit) around zero for both untreated cells and cells exposed to valinomycin. This was true when measured by the distribution of [^{14}C]DMO after silicone oil centrifugation of cells or by flow dialysis (data not shown).

To further analyze the possible involvement of ΔpH in the MGDG/DGDG regulation, the effect of two drugs known to interfere with ΔpH was examined. Gramicidin is a channel-forming quasi-ionophore which permits a flow of protons across the membrane. Upon addition of gramicidin to a growing culture of *A. laidlawii*, there was an immediate decrease in the turbidity of the culture due to swelling of the cells (data not shown). At the concentration of gramicidin used (1 gramicidin molecule per 4000 lipid acyl chains), the cells continued to grow, albeit at a reduced growth rate. This is surprising in view of the extensive leakage as measured by the loss of [^{3}H]uridine-containing material (see Materials and Methods). This leakage made the measurements of ΔpH and $\Delta\psi$ in these cells impossible. With gramicidin, there was only a minor effect on the MGDG/DGDG ratio (Figure 1A). At a comparable valinomycin/lipid acyl chain ratio, there was a profound decrease in the MGDG/DGDG ratio. In other systems, CCCP is known to function as a proton ionophore and uncoupler, dissipating the ΔpH. At a concentration of 10 μM CCCP, no effect was seen on lipid regulation (Figure 5) or on $\Delta\psi$ (Table III).

Effect of TPP^+. The results on cells treated with valinomycin in the presence of different potassium concentrations indicated a correlation between the decrease in the MGDG/DGDG ratio and the corresponding increase in $\Delta\psi$ when valinomycin was added to the cells. To test if there would be an increase in the MGDG/DGDG ratio upon a decrease in $\Delta\psi$, the lipophilic cation TPP^+ was added (unlabeled) at high concentration to the cells. When TPP^+ was added, there was a dose-dependent increase in the MGDG/DGDG ratio (Figure 4A). Addition of similar concentrations

Table III: $\Delta\psi$ in *A. laidlawii* Treated with Carboxylic Ionophores and the Uncoupler CCCP[a]

drug treatment	$\Delta\psi^b$ (mV)	drug treatment	$\Delta\psi^b$ (mV)
control (1% EtOH)	52.4 ± 1.7	nigericin (0.5 nM)	55.0 ± 1.4
monensin (20 nM)	50.4 ± 1.3	nigericin (25 nM)	58.4 ± 1.4
monensin (1 μM)	59.2 ± 1.9	CCCP (10 μM)	51.3 ± 1.6

[a] Determined by [^{3}H]TPP$^+$ accumulation after drug treatment for 15 min. [b] Values are presented with 90% confidence limits.

of NaCl caused a slight decrease in the glycolipid ratio. Substantially larger amounts of NaCl will only marginally decrease the MGDG/DGDG ratio [cf. Christiansson et al. (1985)]. This indicates that the effect of TPP$^+$ is different from common osmotic effects. The lowest concentration of added TPP$^+$ which caused a detectable change in the glycolipid ratio was approximately 100 μM. Concentrations of TPP$^+$ above 100 μM have been said to collapse $\Delta\psi$ due to its characteristics as a lipophilic cation (Rottenberg, 1979; Zaritsky & Macnab, 1981). This was confirmed in *A. laidlawii* (Figure 4B). With 500 μM TPP$^+$, the membrane was depolarized to 42% and 30% of its original value in 15 min and 7 h, respectively. The concentrations of TPP$^+$ used were about 10^4 times higher than those used with valinomycin. However, the amount of TPP bound to the membrane was at the most 1 per 400 fatty acyl chains (data not shown).

Effect of Nigericin and Monensin. Since both TPP$^+$ and valinomycin are considered to affect mainly the electrical potential, it was of interest to examine other ionophores. Nigericin and monensin belong to the group carboxylic ionophores. They both have the ability of cation for proton exchange; i.e., no electrical term is involved in the diffusion reaction. Nigericin is specific for potassium while monensin shows similar but less pronounced specificity for sodium. The addition of nigericin or monensin to a growing cell culture resulted in a decreased MGDG/DGDG ratio (Figure 5) and was accompanied by an increase in $\Delta\psi$ (Table III). These effects were more effectively expressed by nigericin than by monensin. To obtain a similar decrease in the glycolipid ratio and an increase in $\Delta\psi$ as with nigericin, monensin had to be added at a concentration 40 times higher than nigericin (Figure 5 and Table III). In the presence of 25 mM nigericin, the increase in $\Delta\psi$ and the decrease in glycolipid ratio were accompanied by a decrease in the intracellular potassium concentration from 190 to 20 mM. With 1 μM monensin, however, the intracellular potassium concentration was only decreased to 165 mM.

DISCUSSION

Disturbance in Lipid Packing by Valinomycin. It could be argued that the effect of valinomycin on the MGDG/DGDG ratio (Figure 1) is the response to incorporation of a bulky molecule like valinomycin into the membrane. This is probably not the explanation. First, the concentration of valinomycin used was at the most 1 valinomycin molecule per 2000 fatty acyl chains in the membrane. In model systems with gramicidin, a larger ionophore than valinomycin, a ratio of 1/200 (gramicidin/lipid) was needed to induce a change of packing properties and lamellar stability (Van Echteld et al., 1981). In *A. laidlawii*, diethyl ether or tetracaine decreased the MGDG/DGDG ratio to a lesser extent than valinomycin when present at a ratio of 1 molecule per 12–15 lipids (Christiansson et al., 1981). The ratio between the concentrations needed to affect lipid regulation is 200 tetracaines to 1 valinomycin. Furthermore, incorporation of several foreign guest molecules of varying size has revealed that at least 1 molecule per 10 lipid molecules (i.e., 20 acyl chains) is needed for a substantial effect (Wieslander et al., 1985). Second, after valinomycin had been incorporated into the membrane and the effect on the glycolipid ratio had been detected, the MGDG/DGDG ratio could be increased with the addition of external potassium (Figure 1B).

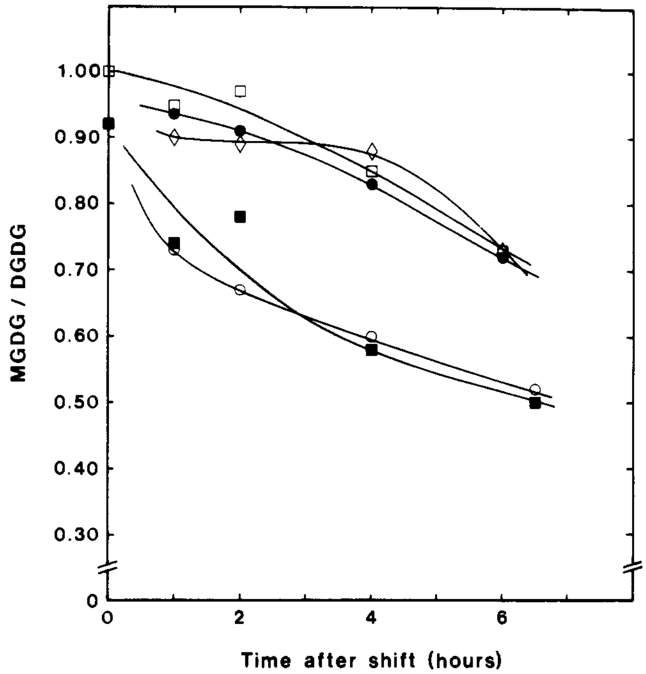

FIGURE 5: Effect of carboxylic ionophores and CCCP on lipid regulation. The conditions of growth and analysis were as in Figure 1: (□) control; (●) monensin (2 nM); (■) monensin (100 nM); (○) nigericin (2 nM); (◇) CCCP (10 μM).

Lipid Regulation and Potassium Flows. It has been reported that protein synthesis among several bacteria has an absolute requirement for the potassium ion (Lubin, 1964). The intracellular concentration of potassium in *A. laidlawii* is about 190 mM. It is reasonable to assume that the valinomycin-induced outflow of potassium would result in a complete or partial inhibition of protein synthesis. In our experiments, the inhibition must be partial as the concentration of valinomycin was chosen to give only a partial reduction in growth rate. Amar et al. (1978) observed a reduction in the incorporation of both fatty acids and amino acids into *A. laidlawii* cells upon valinomycin addition. It is more difficult to explain the effect on lipid regulation by a nonspecific inhibition on protein synthesis. First, in spite of the extensive nonspecific leakage induced by gramicidin (see Materials and Methods), only a reduction of about 8% was observed in the MGDG/DGDG ratio as compared to about 60% reduction with valinomycin at comparable drug/FA ratios (Figure 1A). This nonspecific leakage includes the loss of intracellular potassium (Cho & Morowitz, 1972). Second, the response in the MGDG/DGDG ratio observed with valinomycin was fast. A 10% decrease in the ratio was observed after 10 min, indicating a change in activity rather than a selective change in the amounts of the enzyme in question (Figure 1B). Third, there is no correlation between growth after drug treatment and the glycolipid ratio obtained (Table II). This lack of correlation has been observed for a number of other additives (unpublished experiments).

As expected from its mode of action, valinomycin transported potassium down its concentration gradient and increased $\Delta\psi$. This transport can only occur as long as the transmembrane concentration gradient ($[K^+]_{in} > [K^+]_{out}$) is the dominating part of $\Delta\mu_{K^+}$. An increase in the concentration of extracellular potassium would then be accompanied by a reduced tendency for potassium to be transported out of the cell. As predicted, we observed a reduction in the effect of valinomycin on both hyperpolarization and glycolipid ratio with an increase in the external potassium concentration (Figure 3A,B). Maintenance of hyperpolarization over a long period of time, i.e., 7 h, was accompanied by an effect on lipid regulation that also lasted for the same period. The electrical potential observed across the *A. laidlawii* membrane in the absence of valinomycin is lower than that observed in *Mycoplasma mycoides* (Benyoucef et al., 1981) but is in agreement with that observed (Rottem et al., 1981) in *Mycoplasma gallisepticum* (ca −40 mV). Even though the hyperpolarization obtained after addition of valinomycin is low, it is statistically significant at the concentration shown to affect the MGDG/DGDG ratio (Figures 2 and 3). A hyperpolarization by valinomycin in *A. laidlawii* has also been observed by S. Rottem and M. Shirvan (personal communication).

Effects of TPP⁺. Accompanying the depolarization with high concentrations of TPP^+ was an increase in the MGDG/DGDG ratio (Figure 4A). The added concentration of TPP^+ was approximately 10^4 times higher than that of valinomycin, but the distribution of TPP^+ between the membrane and the bulk solution is at the most 1/400. This means that the actual concentration of TPP^+ in the membrane is only ca. 30 times higher than with valinomycin. For that reason, we do not consider that a packing disturbance is likely due to incorporation of TPP^+ in the membrane. As there is no structural resemblance between TPP^+ and valinomycin, it also seems highly unlikely that they would exert their effect directly on any enzyme or enzyme system in such a way as to cause the observed correlation.

Involvement of ΔpH. In several bacterial and eucaryotic systems, the $\Delta\psi$ and ΔpH are interrelated. A constant $\Delta\mu_{H^+}$ can be maintained even if one of the components is changed. This means that an increase in $\Delta\psi$ is often accompanied by a decrease in ΔpH and vice versa [cf. Padan et al. (1981)]. This is probably not true for *A. laidlawii* as the ΔpH only showed minor fluctuations around zero with or without added valinomycin. Further support for the observed lack of ΔpH was given by the insensitivity of *A. laidlawii* cells to CCCP (Figure 5). That ΔpH is responsible for the observed effect on the MGDG/DGDG ratio is inconsistent with the minor decrease of the glycolipid ratio when gramicidin was added to the culture. Gramicidin permits a free flow of protons across the membrane and invariably dissipates any existing proton gradient.

Lipid Packing and the Electrical Potential. An alternative explanation is based on lipid regulation as a tool for the cell to maintain optimal packing properties in the membrane (Rilfors et al., 1984; Rilfors, 1985; Wieslander et al., 1985). According to this theory, the observed changes in glycolipid ratio can be interpreted as a cellular response to changes in the organization of the membrane matrix, a change inflicted by the effects of the ionophores. In principle, two different mechanisms can be suggested for the effect of the potential. First, according to Robillard & Konings (1982), the balance between dithiol groups and disulfide bridges in proteins or enzymes in membranes can be sensitive to changes in the transmembrane redox potential caused by changes in $\Delta\psi$. Thereby, the activity of, e.g., the enzymes responsible for the MGDG–DGDG balance [DGDG is made from MGDG by glycosylation (Smith, 1969)] could be directly proportional to the magnitude of $\Delta\psi$. Second, a voltage ($\Delta\psi$) across a membrane can interact with both the polar head groups (Crowley, 1973; Lelkes, 1979) and the hydrocarbon chains in the lipids (Sugar, 1979, 1982). According to these authors, this voltage should lead to compression of the bilayer. The compression would tend to shorten and broaden the hydrophobic part of the lipids (Sugar, 1979). Such dimension-related changes in acyl chains shift the phase equilibria of lipids toward nonlamellar phases (Rilfors et al., 1984). By reduction of the MGDG amounts, the cells could then maintain similar phase equilibria when the compression increased (i.e., for hyperpolarization). The considerable decrease in the MGDG/DGDG ratio observed with valinomycin may thus be explained as a response in lipid metabolism to maintain a stable bilayer during compression or hyperpolarization. The increase in electrical potential across the *A. laidlawii* membrane with valinomycin is much lower than the potential required for disruption of the isolating capacity of in vitro membranes (Shchipunov & Drachev, 1982). However, a reversible electrical breakthrough of the membrane can occur long before this voltage. Along similar lines, a depolarization would necessitate an increase in the MGDG/DGDG ratio. The lipids would otherwise have their phase equilibria shifted, due to changes in acyl chain dimensions.

A compression of the membrane, dependent on the transmembrane electrical potential, as a cause for the cell to regulate lipid composition is an attractive theory, particularly in view of the theoretical and in vitro studies mentioned above. So far, however, our work cannot exclude that the observed effects on lipid regulation in *A. laidlawii* in response to ionophores may have other explanations, e.g., electrically neutral transmembrane flow of ions. An attempt to examine the effect of nonelectrical flow of ions was made by the use of the carboxylic ionophores nigericin and monensin. Unexpectedly, there was an increase in $\Delta\psi$ which was accompanied by a decrease in the MGDG/DGDG ratio in the presence of both these ionophores (Figure 5, Table III). These results therefore did not exclude specific ion effects not attributed to electrical phenomena. However, there is no obvious correlation between the intracellular concentration of potassium and the glycolipid ratio. Both nigericin and monensin resulted in approximately the same decrease in the MGDG/DGDG ratio even though monensin only showed a 13% decrease in the intracellular potassium concentration as compared to 90% for nigericin. On the other hand, the extent of hyperpolarization was similar for both of the drugs. This further strengthens the correlation between electrical potential and lipid regulation.

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Correlation between Acetylcholine Receptor Function and Structural Properties of Membranes[†]

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ABSTRACT: Protein–lipid interactions were studied by using *Torpedo californica* acetylcholine receptor (AChR) as a model system by reconstituting purified AChR into membranes containing various synthetic lipids and native lipids. AChR function was determined by measuring two activities at 4 °C: (1) low to high agonist affinity-state transition of AChR in the presence of an agonist (carbamylcholine) in either membrane fragments or sealed vesicles and (2) ion-gating activity of AChR-containing vesicles in response to carbamylcholine. Sixteen samples were examined, each containing different lipid compositions including phosphatidylcholine, cholesterol, phosphatidic acid, phosphatidylethanolamine, asolectin, neutral lipid depleted asolectin, native lipids, and cholesterol-depleted native lipids. Phosphatidylcholines with different configurations of fatty acyl chains were used. The dynamic structures of these membranes were probed by incorporating spin-labeled fatty acid into AChR-containing vesicles and measuring the order parameters. It was found that both aspects of AChR function were highly dependent on the lipid environment even though carbamylcholine binding itself was not affected. An appropriate membrane fluidity was necessarily required to allow the interconversion between the low and high affinity states of AChR. An optimal fluidity hypothesis is proposed to account for the conformational transition properties of membrane proteins. In addition, the conformational change was only a necessary, but not sufficient, condition for the AChR-mediated ion flux activity. Among membranes in which AChR manifested the affinity-state transition, only those containing both cholesterol and negatively charged phospholipids (such as phosphatidic acid) retained the ion-gating activity.

The interactions between membrane proteins and lipids have been studied by various magnetic resonance (Griffith et al., 1982; Devaux, 1983) and fluorescence techniques (London & Feigenson, 1981; Pink et al., 1984). Many recent discoveries have led to the notion that membrane lipids are not only a structural component of cellular compartmentation but also a functionally important element involved in some biological functions of membrane proteins. However, only a few experiments provide a direct link between the specific biochem-

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