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Formation and characterization of planar lipid bilayer membranes from synthetic phytanyl-chained glycolipids

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Abstract

The formability, current–voltage characteristics and stability of the planar lipid bilayer membranes from the synthetic phytanyl-chained glycolipids, 1,3-di-*O*-phytanyl-2-*O*-(β-glycosyl)glycerols (Glc(Phyt)$_2$, Mal$_N$(Phyt)$_2$) were studied. The single bilayer membranes were successfully formed from the glycolipid bearing a maltotriosyl group (Mal$_3$(Phyt)$_2$) by the folding method among the synthetic glycolipids examined. The membrane conductance of Mal$_3$(Phyt)$_2$ bilayers in 100 mM KCl solution was significantly lower than that of natural phospholipid, soybean phospholipids (SBPL) bilayers, and comparable to that of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) bilayers. From the permeation measurements of lipophilic ions through Mal$_3$(Phyt)$_2$ and DPhPC bilayers, it could be presumed that the carbonyl groups in glycerol backbone of the lipid molecule are not necessarily required for the total dipole potential barrier against cations in Mal$_3$(Phyt)$_2$ bilayer. The stability of Mal$_3$(Phyt)$_2$ bilayers against long-term standing and external electric field change was rather high, compared with SBPL bilayers. Furthermore, a preliminary experiment over the functional incorporation of membrane proteins was demonstrated employing the channel proteins derived from octopus retina microvilli vesicles. The channel proteins were functionally incorporated into Mal$_3$(Phyt)$_2$ bilayers in the presence of a negatively charged glycolipid. From these observations, synthetic phytanyl-chained glycolipid bilayers are promising materials for reconstitution and transport studies of membrane proteins.

Keywords: Planar lipid bilayer membrane; Phytanyl-chained glycolipid; Membrane conductance; Membrane stability; Lipophilic ion; Ion channel incorporation

Abbreviations: CL, cardiolipin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPhGG, 1,2-di-*O*-phytanyl-3-*O*-(β-D-glucosyl)glycerol; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; GDNT, glycerol-dialkyl-nonitol tetraether from *Sulfolobus solfataricus*; Glc(Phyt)$_2$, 1,3-di-*O*-phytanyl-2-*O*-(β-D-glucosyl)glycerol; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid; Mal$_N$(Phyt)$_2$, 1,3-di-*O*-phytanyl-2-*O*-(β-glycosyl)glycerol; MGL, main glycolipid from *Thermoplasma acidophilum*; OG, *n*-octyl-β-D-glucoside; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); PS, phosphatidylserine; SBPL, soybean phospholipids; SQDG, sulfoquinovosyldiacylglycerol; TPB^-, tetraphenylborate ion; TPP^+, tetraphenylphosphonium ion; Tris, tris(hydroxylmethyl)aminomethane

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1. Introduction

Planar lipid bilayer membranes have been widely investigated in ion permeation studies via ion carriers, ion channels or ion pumps, and in studies of other membrane-associated processes [1–4]. In these studies, natural lipids with unsaturated fatty acids, e.g., soybean phospholipids (SBPL) or egg yolk phosphatidylcholine (PC) are mostly used. The membranes formed from these types of lipids, however, are not very stable to oxidation, hydrolysis or mechanical stress.

In order to overcome these problems and improve the mechanical stability of planar lipid bilayer membrane, several approaches have been proposed: a blending method with hydrophobic polymers [5], a coating method with polysaccharide derivatives [6], use of polymerizable lipids [7,8], or use of the phospholipids with highly branched hydrophobic chains, phytanic acid, e.g., 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Fig. 1) [9]. In connection to the last approach, diether or tetraether type of lipids with highly branched hydrophobic chains, which are derived from archaeal plasma membranes [10–13], appear also to be interesting lipids for the construction of the stable bilayer (diether lipid) or monolayer (tetraether lipid) membranes [12–25]. These archaeal lipid membranes are thought to be more effective than DPhPC membranes in certain cases in which the pH of the aqueous phase is extremely low or high. Although DPhPC bilayers are very stable under mechanical stress [9], the ester linkages between acyl chains and glycerol backbone as well as between headgroup and glycerol backbone in DPhPC molecule are relatively susceptible to hydrolysis under acidic or alkaline conditions.

Unfortunately, archaeal lipids are limited in available amount and purity, therefore relatively few studies concerning membrane properties have been performed from the viewpoint of chemical structures of this type of lipid molecules. Thus, if possible, synthetic model archaeal lipids seem attractive materials in the preparation and investigation of planar lipid bilayer membranes.

In the previous paper, we synthesized a series of diether type phytanyl-chained glycolipids, 1,3-di-*O*-phytanyl-2-*O*-(β-glycosyl)glycerols ($Glc(Phyt)_2$, $Mal_N(Phyt)_2$ ($N = 2, 3, 5$; see Fig. 1)), as model archaeal glycolipids, and investigated the phase behavior of lipid/water dispersion systems in terms of the size and geometry of the saccharide headgroup [26,27].

In this paper, we investigated the formability of the planar lipid bilayer membranes from the synthetic phytanyl-chained glycolipids, their current–voltage characteristics, and the membrane stability against long-term standing and an external electric field change. By comparing the results with other lipids, SBPL and DPhPC, we discussed the effect of molecular structures on membrane properties. Furthermore, we performed a preliminary experiment to assess applicability of the phytanyl-chained glycolipid bilayers to the functional incorporation of membrane proteins, employing the channel proteins derived from octopus retina microvilli vesicles.

2. Materials and methods

2.1. Materials

$Glc(Phyt)_2$, $Mal_N(Phyt)_2$ ($N = 2, 3, 5$; Fig. 1 shows the chemical structures of $Glc(Phyt)_2$ and $Mal_3(Phyt)_2$) were synthesized as described [26,27]. SBPL was purchased from Associated Concentrates (Woodside, NY, USA) and used after partial purification by the method of Kagawa and Racker [28]. The purified SBPL comprised 40 mol% PC, 30 mol% PE, 5 mol% CL, 5 mol% PI, 5 mol% PS and 15 mol% PA, determined by an Iatroscan MK-5 TLC/FID analyzer (Iatron Laboratories, Tokyo, Japan). No free fatty acid was detected in the stock solution. DPhPC was purchased from Avanti Polar Lipids (Birmingham, AL, USA) and sulfoquinovosyldiacylglycerol (SQDG) was purchased from Lipid Products (South Nutfield, Surrey, UK). They were used as received and no free fatty acids were detected in both stock solutions. All of the lipid stock solutions were kept in the freezer at −20°C. *n*-Octyl-β-D-glucoside (OG), tetraphenylphosphonium (TPP^+) chloride and tetraphenylborate (TPB^-) sodium salt were purchased from Dojindo (Kumamoto, Japan). All other reagents were of analytical reagent grade and obtained from Wako Pure Chemical Industries (Osaka, Japan). Microvilli vesicles prepared from octopus retina [29] were kindly provided by Dr. H. Hirata, Himeji Institute of Technology, Hyogo, Japan.

Water was purified with a Milli-Q SP UF system (Millipore Corp., Tokyo, Japan) and its conductivity was around 18 MΩ/cm.

2.2. Surface pressure–area isotherm measurements

Surface pressure–area isotherm measurements were carried out by using a Teflon Langmuir trough (surface area: 225.6 cm^2; volume: 260 cm^3) with a Model HBM surface film balance (Kyowa Kaimen Kagaku, Tokyo, Japan) at 23 ± 0.1°C. Surface pressure was measured by a Wilhelmy technique using a sandblasted platinum plate. Lipid monolayers were prepared by spreading a chloroformic solution of lipids onto the subphase of 100 mM KCl unbuffered solution. Molecular occupied area was varied by means of the successive addition method [30]. Accuracy for surface pressure was determined to be ± 0.05 mN/m.

2.3. Preparation of planar lipid bilayer membranes

Planar lipid bilayer membranes were formed by the folding method [31,32] in the aperture (diameter: 100–250 μm) located in a Teflon thin sheet (25 μm thick, Yellow Springs Instruments, Yellow Springs, OH, USA). The apertures were made by electrical discharge and their sizes were measured with an optical microscope. The estimated error of the aperture area was of the order of 5%. Two Teflon chambers (internal volume: approx. 2.0 ml) separated by the Teflon sheet were filled with 100 mM KCl unbuffered (pH around 6) or 100–250 mM KCl/10 mM Hepes–Tris (pH 7.4) buffer solution. For TPP^+ and TPB^- permeation measurements, 100 mM NaCl unbuffered solution was used instead of KCl solution. Before the formation of bilayer membranes, the aperture in the Teflon thin sheet was treated with 1% v/v of hexadecane dissolved in hexane or in chloroform/methanol (2:1 v/v). Appropriate amount of lipid dissolved in chloroform/methanol (2:1 v/v) or hexane was placed onto the surface of an aqueous solution, and allowed to stand for at least 15 min and subsequently, water level was raised to form the planar lipid bilayer membrane in the aperture. Prior to the electric measurements, the formed bilayer was allowed to stand for at least 10 min.

For incorporation of the octopus retina channels into planar lipid bilayer membranes, the fusion method [1,3,4] was adopted. The microvilli vesicles solubilized with 2% w/v OG solution were added to one (*cis* side and this side was grounded) of the gently stirred aqueous phases (250 mM KCl/10 mM Hepes–Tris (pH 7.4) buffer solution or 400 mM gluconate (pH 7.4) buffer solution) after formation of the planar lipid bilayer membrane. In order to enhance the fusion, osmotic gradient that leads to the swelling of the microvilli vesicles and the external voltage of +150 mV (*cis* side) was applied. In certain cases, a negatively charged lipid, SQDG was added into bi-

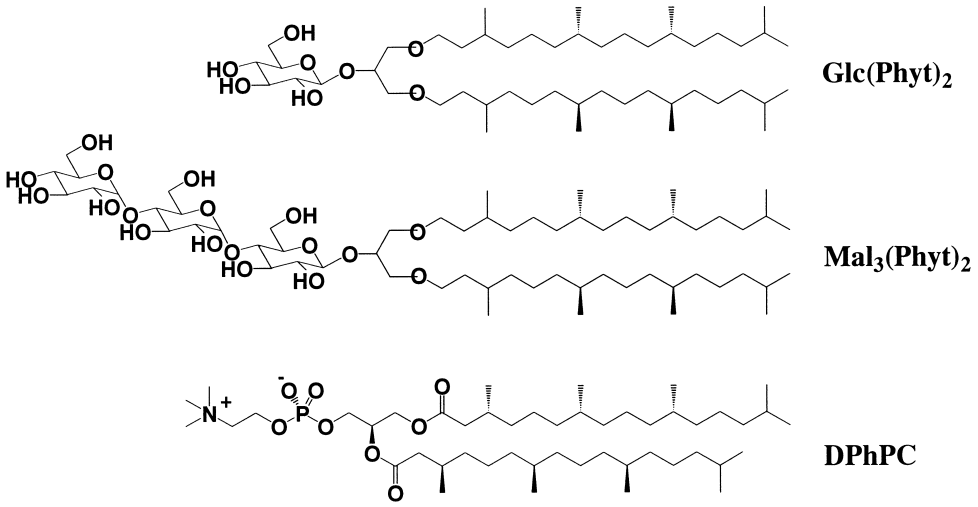

Fig. 1. Chemical structures of 1,3-di-*O*-phytanyl-2-*O*-(β-D-glucosyl)glycerol ($Glc(Phyt)_2$), 1,3-di-*O*-phytanyl-2-*O*-(β-D-maltotriosyl)glycerol ($Mal_3(Phyt)_2$) and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC).

layer as much as 5 mol% and hexane was used as a solvent for this membrane preparation. The added OG induced some baseline noises in the time course of current and a non-specific increase in conductance in bilayers at the final OG concentration of less than 0.02% w/v. After perfusion of buffer, OG-induced noises were reduced, indicating the removal of the OG molecules in the bilayer as well as the removal of free microvilli vesicles.

2.4. Electrical measurements

Teflon chambers and preamplifier were put in the grounded shield box on a vibration absorbing base. A couple of Ag/AgCl electrodes via KCl salt bridges was used to apply external voltages and to monitor current changes. For TPP^+ and TPB^- permeation measurements, the use of the KCl salt bridges was omitted. Electrical signals were preamplified with a current to voltage converter equipped with a 50 GΩ feed-back resistor. The converted signals were amplified with a CEZ-2300 patch/whole cell clamp amplifier (Nihon Koden, Tokyo, Japan), displayed on a VC-6045 digital storage oscilloscope (Hitachi, Tokyo, Japan) via a low-pass filter, and recorded on a videotape recorder or a chart recorder. Membrane capacitance, C_m, was measured by applying a triangular wave (usually 20 Hz and 4–25 mV peak to peak) with an Iwatsu SG-4101 or an HP 33120A function generator. Membrane conductance, G_m, was determined by applying a rectangular wave and then eliminating the capacitive currents. Electrical rupture of the membranes was induced by applying the linearly elevating electric fields at the rate of 8.0 mV/s (aperture diameter: 220–250 μm). Membrane rupture potential, V_r, was defined as the voltage at which the current abruptly increases and no longer recovers the original membrane conductance. All measurements were performed at ambient temperatures (22–25°C).

3. Results and discussion

3.1. Formability of planar lipid bilayer membranes

All of the phytanyl-chained glycolipids as well as DPhPC gave liquid-expanded monolayers at the air–100 mM KCl aqueous solution interface at 23°C, as shown in Fig. 2. These results indicate that all of the monolayers examined are in a fluid state under the present conditions. Since such a state is preferable for the formation of stable planar lipid bilayer membranes [33], the formability of planar lipid bilayers was examined under the same conditions and the monolayers were always set at their highest surface pressures (collapse state). Among the phytanyl-chained glycolipids examined, it was found that $Mal_3(Phyt)_2$ easily formed planar lipid bilayer membranes. From the measurements of the capacitive current passing through the $Mal_3(Phyt)_2$ bilayer, the membrane capacitance, C_m, was evaluated as 0.6–0.7 μF/cm^2, indicating the existence of a solvent-free single bilayer in the aperture. Since the contribution of the electrical double layer at the membrane surface to C_m is negligibly small under the higher salt concentration (100 mM KCl) conditions [1], the geometric thickness of the bilayer, d, can be estimated from Eq. 1 to a fairly good approximation:

$$d = \varepsilon_0 \, \varepsilon_m / C_m \quad (1)$$

where ε_0 is the permittivity of free space 8.854 pF/m and ε_m is the dielectric constant of hydrophobic chain region. For ε_m, the average value of a long chain hydrocarbon, $\varepsilon_m = 2.1$ [34] was used. The geometric thickness for $Mal_3(Phyt)_2$ bilayer was 2.9 ± 0.2 nm, in moderate accordance with the previously estimated thickness (2.55 nm) of hydrophobic chain region of $Mal_3(Phyt)_2$ bilayer determined by small-angle X-ray scattering measurements [26].

In contrast to $Mal_3(Phyt)_2$, $Mal_2(Phyt)_2$ and $Glc(Phyt)_2$ did not successfully form the stable single bilayers, while they gave more condensed monolayers than $Mal_3(Phyt)_2$ and their isotherms were analogous to that of DPhPC, as can be seen in Fig. 2. Our previous data showed that $Mal_2(Phyt)_2$ and $Glc(Phyt)_2$ cannot form lamellar phase in aqueous dispersions but form inverted hexagonal liquid–crystalline phase [26] or inverted cubic phase [27] as a stable phase at ambient temperatures. According to the 'lipid packing concept' [35,36], the phytanyl-chained lipids bearing the smaller headgroups are thought to prefer negative-curvature aggregates (e.g., inverted hexagonal liquid–crystalline) to almost zero-curvature (planar bilayer) or positive curvature

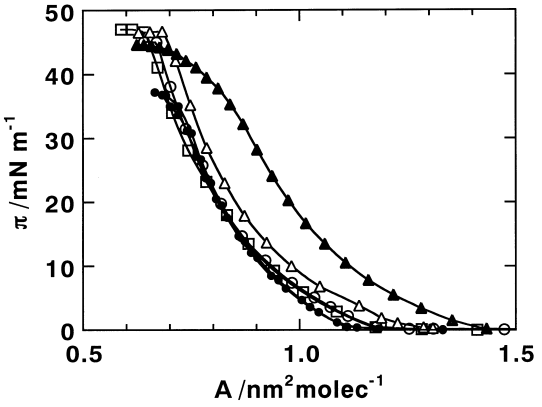

Fig. 2. Surface pressure–area (π–A) isotherms for phytanyl-chained glycolipids and DPhPC at the air–water interface. Subphase was 100 mM KCl unbuffered solution (pH around 6). Temperature was 23 ± 0.1°C. (●) Glc(Phyt)$_2$; (○) Mal$_2$(Phyt)$_2$; (△) Mal$_3$(Phyt)$_2$; (▲) Mal$_5$(Phyt)$_2$; (□) DPhPC.

ones (e.g., normal hexagonal liquid–crystalline). Consequently, these phytanyl-chained lipids cannot be expected to pack into planar lipid bilayer membranes under the present conditions. Stern et al. [20] reported that the planar lipid bilayer membranes from synthetic 1,2-di-*O*-phytanyl-3-*O*-glucosylglycerol (DPhGG), of which structure is very close to that of Glc(Phyt)$_2$, are less stable compared to DPhPC membranes. This result can be also understood in terms of the molecular shape of lipid. Since the headgroup size of DPhGG is rather small compared to that of Mal$_3$(Phyt)$_2$ and there is unbalanced lateral constraints between the headgroup region and the hydrophobic central one in a zero-curvature bilayer, the stability of DPhGG bilayers is expected to be reduced.

Mal$_5$(Phyt)$_2$ can form the lamellar phase in aqueous dispersion as a stable phase at ambient temperatures [26]; however, this glycolipid also failed to form stable planar lipid bilayer membranes. This unexpected result might be attributed to the fact that the Mal$_5$(Phyt)$_2$ molecule prefers gentle positive curvature (curved bilayer) to almost zero-curvature (planar bilayer) due to the larger headgroup of the Mal$_5$(Phyt)$_2$ molecule. The isotherm for Mal$_5$(Phyt)$_2$ in Fig. 2 shows that the more expanded monolayer was formed at the air–water interface as compared to the Mal$_3$(Phyt)$_2$ monolayer, suggesting that the cross-sectional area of a headgroup of Mal$_5$(Phyt)$_2$ is larger than that of hydrophobic chains.

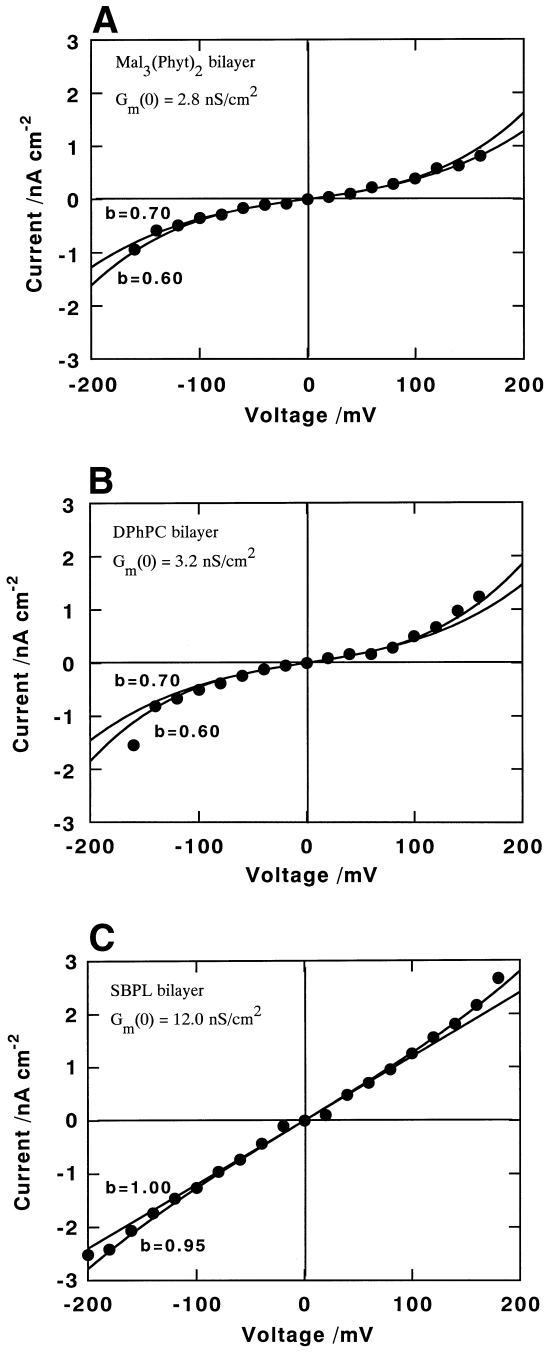

Fig. 3. Current–voltage relationships for various lipid bilayer membranes. (A) Mal$_3$(Phyt)$_2$; (B) DPhPC; (C) SBPL. Planar lipid bilayer membranes were formed in 100 mM KCl unbuffered solution (pH around 6). Temperature was 23°C. Surface areas of the membranes were 4.0–4.9 × 10^{-2} cm^2. The fitting curves were calculated from Eq. 2 changing the value of b. The membrane conductance at 0 mV, $G_m(0)$, was practically evaluated from the conductance at ±40 mV.

Table 1
Electric characteristics of various planar lipid bilayer membranes

Lipid	Method[a]	Aqueous phase	Conductance, nS/cm^2	b[b]	Capacitance, μF/cm^2 (thickness)	Membrane rupture potential, mV	Ref.
Diethers							
$Mal_3(Phyt)_2$	F	0.1 M KCl (pH 6)	2.8 (23°C)	0.7	0.6–0.7 (2.7–3.1 nm)	344 ± 63 (n = 20)[c]	This work
DPhGG	P (squalene)	1 M KCl	Unstable	–	0.7 (2.6–3.0 nm)	–	[20]
Tetraethers							
GDNT	P (squalene)	KCl	< 10 (65°C)	–	0.7 (2.5–3.0 nm)	–	[18]
	P (squalene)	0.1 M KCl (pH 6)	33–100 (46°C)	–	0.70	–	[19]
MGL	P (decane)	1 M KCl	–	–	0.744 (2.5–3.0 nm)	–	[20]
Phospholipids							
SBPL	F	0.1 M KCl (pH 6)	12 (23°C)	0.95	0.6–0.7	293 ± 44 (n = 15)	This work
	F	0.5 M KCl (pH 6.2)[e]	(5–12) × 10^3 (21°C)	–	0.74	–	[8]
Egg yolk PC	F	0.1 M KCl (pH 7)[g]	–	–	0.6	V_c = 280 ± 70[d]	[49]
DOPC	P (decane)	0.1 M NaCl (pH 7)[f]	40 ± 10 (24°C)	–	–	210 ± 10	[38]
DPhPC	F	0.1 M KCl (pH 6)	3.2 (23°C)	0.6	0.6–0.7	411 ± 42 (n = 6)	This work
	F	0.1 M KCl (pH 7)[g]	–	–	–	V_c = 390 ± 70[d]	[49]
	F	0.5 M KCl (pH 6.2)[e]	70–100 (21°C)	–	0.78	–	[8]
	P (decane)	1 M KCl	10 (25°C)	–	0.35	–	[6]

[a] F, folding method; P, painting method (solvent used).
[b] Fraction of the membrane spanned by the minor base of the trapezoid (see Eq. 2).
[c] Mean values of n membranes.
[d] Measured under current-clamp conditions.
[e] Containing 1 mM Pipes.
[f] Containing 1 mM $CaCl_2$.
[g] Containing Tris–HCl.

In any event, these results indicate that the headgroup geometry as well as hydrophobic chains in the fluid state are considerably important in preparing stable planar lipid bilayer structures.

3.2. Current–voltage characteristics of planar lipid bilayer membranes

The steady-state transmembrane currents were recorded for each 20 mV increment of the applied voltage. The membrane conductance at 0 mV, $G_m(0)$, was practically evaluated from the conductance at ± 40 mV where the current–voltage relationship can be considered linear [37], and the data points were fitted by simulation curves calculated from Eq. 2 as described below. Fig. 3 and Table 1 show the current–voltage characteristics for three kinds of lipid bilayer membranes, which were formed in 100 mM KCl unbuffered solution (pH around 6) at 23°C. Obviously, $Mal_3(Phyt)_2$ (Fig. 3A) and DPhPC (Fig. 3B) bilayers showed comparable conductances as well, which are rather low compared with the SBPL (Fig. 3C) bilayer. Although the reported conductance data for various planar lipid bi-

layer membranes (Table 1) are significantly scattered due to the differences in the membrane preparation method and/or measurement conditions, the bilayer membranes from the lipids with branched chains (diethers, tetraethers, DPhPC) tend to exhibit the lower conductances; this implies that the bilayer membranes from the lipids with branched chains show lower permeability and higher barrier property compared with the membranes from conventional phospholipids. It has been reported that liposome membranes composed of archaeal lipids show much lower permeability against solutes, such as H^+ or hydrophilic dyes [12,13,21–23,25]. The present finding is in accord with the conclusions drawn from the earlier studies on liposome membranes. These results can be explained by the characteristic structure of the branched chain, in which bulky methyl branches may prevent the formation of transient permeation paths for the small ions or the formation of hydrogen-bonded water chains in the hydrophobic chain region of the bilayer. Therefore, it can be considered that the rate-limiting step for small ion permeation is the translocation of the ions through a hydrophobic chain region rather than a headgroup region, as supported by the comparable conductances of $Mal_3(Phyt)_2$ and DPhPC bilayers.

The current–voltage fitting curves were found to be symmetric and to deviate from linearity at higher voltages depending on the type of lipid, as seen in Fig. 3. This type of characteristic has been reported in many cases, e.g., H^+/OH^- voltage-driven permeation [37], inorganic ion permeation [38], ion carrier-mediated transport [39,40] and lipophilic ion transport [41] through the planar lipid bilayer membranes, which were often ascribed to the trapezoidal potential energy barrier in the membrane against the charged solute permeation [37–39,42]. In this case, the current density strongly depends on the height of this potential energy barrier. The normalized conductance for dielectrically symmetrical bilayer membranes can be obtained by Eq. 2 [37–39]:

$$G_m(V)/G_m(0) = b\sinh(eV/2kT)/\sinh(beV/2kT) \quad (2)$$

where $G_m(V)/G_m(0)$ is the ratio of the conductance at voltage V to the conductance at 0 mV, b is the fraction of the membrane spanned by the minor base of the trapezoid. e, k and T have their usual meanings. For $Mal_3(Phyt)_2$ and DPhPC bilayers, a value of $b = 0.6$–0.7 gives the best fit, as shown in Fig. 3A and B. For SBPL bilayer, the fitting curve is almost linear ($b = 0.9$–1.0) over the examined voltage region, as shown in Fig. 3C. Since $(1-b)/2$ is a measure of displacement of the barrier corner toward the bilayer center due to the reduction of the Born self-energy of an ion by attraction of an ion to its image, the estimated value b may give information concerning the bilayer–water interface dielectric structure [39]. Judging from the b values for three kinds of membranes, the bilayer–water interface dielectric structure of $Mal_3(Phyt)_2$ membrane is analogous to that of DPhPC, regardless of the different headgroup structure.

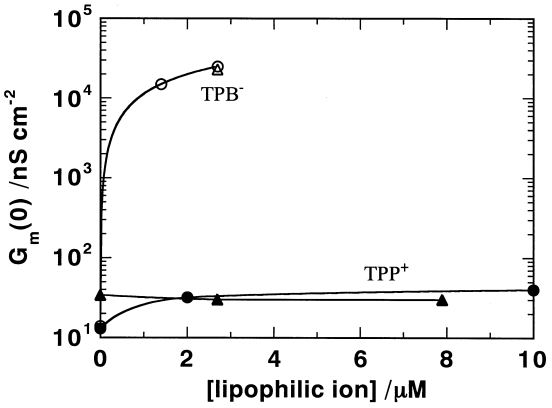

Fig. 4. Effects of lipophilic ions, TPP^+ and TPB^- on the membrane conductances at 0 mV, $G_m(0)$, of $Mal_3(Phyt)_2$ and DPhPC bilayer membranes. triangles, $Mal_3(Phyt)_2$; circles, DPhPC. Closed symbols, TPP^+; open symbols, TPB^-. Planar lipid bilayer membranes were formed in 100 mM NaCl unbuffered solution (pH around 6). Temperature was 22–25°C. Surface areas of the membranes were 3.0–4.0×10^{-2} cm^2.

In addition to observations of small ion permeation behavior through the branched-chain lipid bilayers, measurements of current–voltage characteristics of lipophilic ions through the bilayers can also give the additional information concerning the bilayer–water interface dielectric structure. By comparing the permeation rates of the lipophilic cation, TPP^+ and the lipophilic anion, TPB^-, through the bilayers, contribution of molecular structure of lipid headgroup to a bilayer membrane dipole potential [41–44] can be considered. In Fig. 4, variations of $G_m(0)$ of $Mal_3(Phyt)_2$ and DPhPC bilayer mem-

branes are shown as a function of the concentration of lipophilic ions. For TPP^+, $G_m(0)$ did not significantly change upon the addition of small amount of lipophilic ion and was practically constant in the examined concentration region in both bilayer membranes. For TPB^-, however, if only a slight amount of ion was added, greater than 10^3-fold increases in $G_m(0)$ were observed in both bilayer membranes. It is noted that the values of membrane conductance obtained in the presence of TPB^- were not so accurate due to the significant capacitive currents even at lower voltages and the instrumental limitation of current detection.

It has been reported that TPB^- has much greater permeability than TPP^+ [42–44]. The difference in permeation rate of TPB^- and TPP^+, that is, anion-selective permeation, has been explained in terms of a bilayer membrane dipole potential model [42–44]. On the basis of this proposed model, the source of dipole potential is ascribed to the dipole organization of molecules at the bilayer–water interface, with the most significant contribution coming from the carbonyl groups in glycerol backbone, lipid headgroup, and surface water molecules [42–44]. The observation in this study showed that the permeation characteristics of lipophilic ions through the bilayers of branched-chain type lipid appear to be analogous to each other in both cases of $Mal_3(Phyt)_2$ as well as DPhPC, suggesting there is no significant difference in the dipole potential barrier between the two types of branched-chain lipids. On the basis of the present result, it can be presumed that the carbonyl groups in the glycerol backbone of the lipid molecule are not necessarily required for the total dipole potential barrier against cations in the $Mal_3(Phyt)_2$ bilayer. The anion-selective permeation through the $Mal_3(Phyt)_2$ bilayer may be accounted for by the saccharide headgroup bearing many hydroxyl dipoles, making up the lack of the carbonyl groups and contributing to the total dipole potential barrier. Therefore, it can be considered that the bilayer–water interface dielectric structure of $Mal_3(Phyt)_2$ membrane is analogous to that of DPhPC, regardless of the headgroup structure.

3.3. Stability of planar lipid bilayer membranes

For the stability measurements, we selected the bilayers which had higher capacities (>0.6 μF/cm^2) and showed reasonable currents without fluctuations at the applied voltage of 100 mV. The long-term stability of the planar lipid bilayer membranes was checked at the applied voltage of 0 mV. $Mal_3(Phyt)_2$ bilayers were considerably stable, in the best preparation case, for at least 3 days, whereas SBPL bilayers were usually stable only for less than 8 h under the same conditions.

Next, the membrane stability against the external electric field change was evaluated with the applied voltage at which the membrane rupture occurs, V_r. As summarized in Table 1, the values of V_r for DPhPC (411 ± 42 mV) and $Mal_3(Phyt)_2$ (344 ± 63 mV) bilayers are higher than that for SBPL (293 ± 44 mV). This result implies that DPhPC and $Mal_3(Phyt)_2$ bilayers are considered to be more stable than the SBPL bilayer against the external electric field change.

It has been reported that a significant increase in conductance of pure lipid bilayer membranes is often observed due to reversible or irreversible electrical membrane breakdown at higher applied potentials [45,46]. According to the data reported by Shchipunov and Drachev [47], the voltage scanning rate of 8.0 mV/s as an external electric field change in this study is slow enough that the membrane rupture is mainly caused by the transient pores or defects in a bilayer. At such low voltage scanning rates, membranes from many types of lipids have been reported to break down irreversibly at 100–300 mV [38,47,48]. In addition, Robello and Gliozzi [49] have reported the critical potential at which conductance transition occurs due to the formation of aqueous pores, V_c, under current-clamp conditions. The V_c values reported are listed in Table 1. The value of V_c for the DPhPC bilayer is very close to the value of V_r for this lipid. Similarly, the value of V_c for the egg yolk PC bilayer is very close to the value of V_r for SBPL. From these comparisons, the present electric behavior of the bilayers is presumed to be the aqueous pore formation in the bilayers. In this context, the aqueous pore formation in the bilayers seems to be much suppressed for $Mal_3(Phyt)_2$ and DPhPC, compared with SBPL. According to the energetic theory that explains the aqueous pore-induced membrane breakdown [46,50,51], the pores with an over-critical radius are responsible for the irreversible

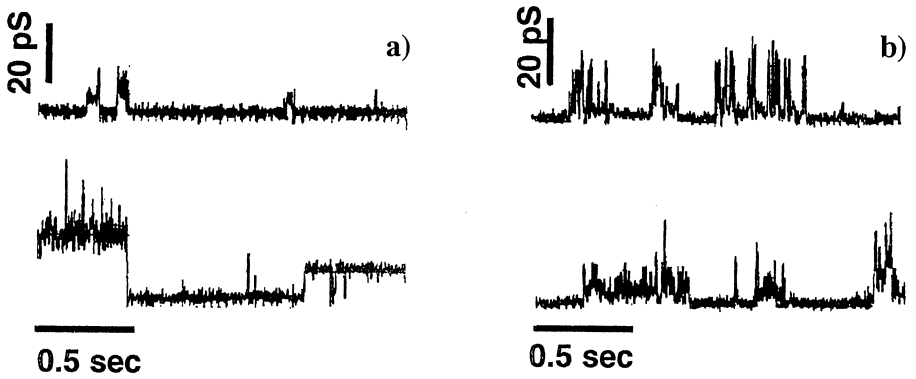

Fig. 5. Typical current fluctuations of the ion channels of octopus retina microvilli vesicles in planar lipid bilayer membranes. (a) SBPL; (b) $Mal_3(Phyt)_2$/SQDG (95:5, mol/mol). Planar lipid bilayer membranes were formed in 250 mM KCl/10 mM Hepes–Tris (pH 7.4) buffer solution. The applied potential was +100 mV. Temperature was 23°C.

membrane breakdown, and the energy barrier height of pore formation can be related to the pore edge line tension and the surface tension of a bilayer. Thus, the bilayers with higher pore edge line tension may require a higher applied voltage to induce membrane rupture [46,50,51]. Although we cannot evaluate the pore edge line tension and surface tension for lipid bilayers at the present stage, it seems likely that the higher stability of phytanyl- or phytanoyl-chained lipid bilayer membrane to the external electrical field change may be ascribed to the higher pore edge tension, that is, the higher lateral cohesion in this type of membrane.

These findings are consistent with the previously reported results showing that liposome membranes composed of archaeal lipids are very stable over a broad range of conditions, including temperature and pH [12,13,21–24].

3.4. *Incorporation of octopus retina ion channels into planar lipid bilayer membranes*

Based on the above results, the phytanyl-chained glycolipid bilayers are expected to be promising for biochemical studies over functional reconstitution of membrane proteins because of chemical stability as well as membrane stability. Therefore, we performed a preliminary experiment to assess applicability of the phytanyl-chained glycolipid bilayers to the functional incorporation of membrane proteins. In this work, we employed the channel proteins derived from octopus retina microvilli vesicles. It has been reported that the ion channels of microvilli vesicles are heterogeneous, showing specific conductances ranging from 10 pS to 200 pS [29].

In the case of the bilayer comprising $Mal_3(Phyt)_2$ alone, however, the fusion with vesicles did not occur, which was indicated by the fact that no current fluctuation was observed even after the addition of microvilli vesicles. In contrast to the glycolipid bilayer, the functional incorporation of ion channels into SBPL bilayer proceeded successfully, as can be seen in Fig. 5a. SBPL contains a certain amount of negatively charged lipids and it may promote fusion with vesicles [1,3,4]. Therefore, it was expected that the use of negatively charged lipids might promote the incorporation of membrane proteins. For this reason, the natural sulfoglycolipid SQDG was used as a negatively charged lipid. In the case of the $Mal_3(Phyt)_2$ bilayer containing 5 mol% SQDG, the current fluctuations were successfully observed under voltage-clamp conditions even after the removal of OG, as shown in Fig. 5b, suggesting the functional incorporation of ion channels into the bilayer. It is noted that the presence of SQDG in $Mal_3(Phyt)_2$ bilayer did not enhance the current fluctuations prior to the addition of OG-solubilized microvilli vesicles.

This preliminary experiment demonstrated, to some extent, applicability of the phytanyl-chained glycolipid bilayer as matrix materials to the reconstitution of membrane proteins, although further studies with these ion channels and other membrane proteins should be required. We are now progressing on a study involving functional reconstitution of membrane proteins into our synthetic phytanyl-chained glycolipid vesicles.

3.5. Concluding remarks

The planar lipid bilayer membranes were successfully formed from the synthetic phytanyl-chained glycolipid bearing a maltotriosyl group ($Mal_3(Phyt)_2$) by the folding method. The membrane conductance of $Mal_3(Phyt)_2$ bilayers in 100 mM KCl aqueous solution was significantly lower than that of natural phospholipid, SBPL bilayers and comparable to that of DPhPC bilayers. Comparison of the current–voltage characteristics of $Mal_3(Phyt)_2$ bilayer membranes with those of DPhPC ones supported the view that the rate-limiting step for small ion permeation is the translocation of ion across a hydrophobic chain region rather than a headgroup region. From the permeation measurements of lipophilic ions through $Mal_3(Phyt)_2$ and DPhPC bilayers, it could be presumed that the carbonyl groups in glycerol backbone of the lipid molecule are not necessarily required for the total dipole potential barrier against cations in $Mal_3(Phyt)_2$ bilayer. The stability of the phytanyl-chained glycolipid bilayer membranes against long-term standing and external electric field change was rather higher than that of SBPL. In addition, $Mal_3(Phyt)_2$ bilayer was considered to be more chemically stable with respect of the linkages among glycerol, hydrophobic chains and headgroup (ether linkages versus ester linkages), compared with the DPhPC bilayer. Furthermore, the channel proteins derived from octopus retina microvilli vesicles were successfully incorporated into $Mal_3(Phyt)_2$ bilayers in the presence of the negatively charged glycolipid, SQDG.

From these observations, it is concluded that synthetic phytanyl-chained glycolipid bilayers are promising materials for reconstitution and transport studies of membrane proteins, as well as for application to membrane protein-immobilized bilayer type bioreactors and/or biosensors, owing to membrane stability.

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