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Interaction of peptides and proteins with bacterial surface glycolipids: a comparison of glycosphingolipids and lipopolysaccharides

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The bacterial cell wall of Gram-negative bacteria consists, in addition to the cytoplasmic membrane, of another permeability barrier, the outer membrane. The lipid distribution between both sides of this membrane is strictly asymmetric. The outer leaflet is made up of glycolipids, usually lipopolysaccharides. In *Sphingomonas* spp glycosphingolipids were found to substitute for lipopolysaccharides. In this review, it is shown by an electrophysiological approach that glycosphingolipid can replace lipopolysaccharide with respect to its function as antigenic surface structure as well as to its contribution to the diffusion barrier properties of the outer membrane. This review is focused on: (i) the function of porins, as examples of transmembrane proteins, in the different glycolipid environments; (ii) the interaction of polymyxin B with the outer membrane, as an example of polycationic antibacterial peptides; and (iii) the activation of the human complement system by lipopolysaccharides and glycosphingolipids.

Keywords: outer membrane; glycolipid; reconstituted membranes; porins; complement activation; polycationic antibiotics

Membranes, in general, constitute the boundary between a cell or a cell compartment and its environment. They function as permeability barriers, maintain constant ion gradients across the membrane and guarantee a controlled steady state of fluxes in the cell. Furthermore, the vast majority of cell membranes carry recognition sites for interaction/communication with other cells. In the case of bacteria, these recognition sites are also targets for components of the immune system and exogenous substances like antibiotics. For these various functions to work properly, a particular lipid composition on each side and distribution between both sides of the lipid bilayer is required.

The cell envelope of Gram-negative bacteria is composed of the cytoplasmic membrane, the peptidoglycan layer, and the outer membrane, which represents an additional permeability barrier and which is strictly asymmetric with respect to its lipid composition. Whereas the inner leaflet of the outer membrane contains only phospholipids, the outer leaflet is usually composed of lipopolysaccharides (LPS), which consist of an oligo- or polysaccharide moiety and a covalently linked lipid component, called lipid A, anchoring the LPS in the outer membrane [54]. In wild-type strains, the polysaccharide portion consists of an O-specific chain and the core oligosaccharide. Rough mutant strains do not express the O side chain, but retain core oligosaccharides of varying length. The LPS of various rough mutants are characterized by chemotypes in the sequence of decreasing length of the core sugar as Ra (complete core), Rb, Rc, Rd, and Re, the latter representing the minimal structure of LPS consisting of only lipid A and two 2-keto-3-deoxyoctonate (Kdo) monosaccharides. Lipid A is composed of a β-glucosaminyl-(1→6)-α-D-glucosamine disaccharide backbone, which is phosphorylated in positions 1 and 4′ of the disaccharide backbone and carries—for *Enterobacteriaceae*—six or seven (hydroxy) fatty acid residues, which are either ester- or amide-linked (for a review see [89]).

It was found in earlier studies that for the strictly aerobic Gram-negative rod *Sphingomonas paucimobilis* [86] (formerly *Flavobacterium devorans* [87] and *Pseudomonas paucimobilis* [26]), the attempt to extract an LPS by the phenol/water [77] or the phenol/chloroform/petrol ether method [22] was not successful [30]. Instead, a glycolipid with unexpected and unusual structural features was obtained. This 'lipid A-type' glycolipid carried the expected D-glucosamine; however, (*R*)-3-hydroxylated fatty acid residues were absent. Kawahara *et al* [31] succeeded in elucidating the complete chemical structure of the glycosphingolipid (GSL) from *S. paucimobilis* IAM 12576. The hydrophobic portion was found to be heterogeneous with respect to the dihydrosphingosine residue but was quantitatively substituted by a (*S*)-2-hydroxymyristic acid in amide linkage. The oligosaccharide portion of the two main fractions, GSL-4A and GSL-1, consists of a Man-Gal-GlcN-GlcA tetrasaccharide, and of a GlcA monosaccharide, respectively (for details see also pp 408–413 of this issue).

In this review, we focus on the differences and similarities of LPS and GSL as constituents of the outer membrane of Gram-negative bacteria with emphasis on our own investigations on the function of porins, the glycolipid-induced activation of the complement (C) system, and the interaction with antibacterial polycationic peptides.

A detailed characterization of the functions of the bacterial outer membrane is hampered by its complexity.

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Therefore, the reconstitution of simpler model systems, eg, as a first step that of the unmodified lipid matrix being composed on one side of glycolipids (LPS or GSL) and on the other side of phospholipids, is a feasible approach to study the role of the glycolipid leaflet of the outer membrane in its potential interaction with membrane active substances like drugs, detergents, and components of the immune system and for its influence on the function of transmembrane proteins. Based on the Montal-Mueller technique [41], we have established a reconstitution system of the outer membrane as an asymmetric glycolipid/phospholipid bilayer [61]. With this model system using electrophysiological techniques, all those processes can be studied, which lead to changes of the electrical parameters of the membrane such as membrane conductivity, membrane capacitance, and transmembrane potential (for review see [82]). By these means, we have been able to obtain information on processes such as porin incorporation [78,79], C activation [46,58,79], and interaction with antibacterial polycationic peptides including polymyxin B (PMB) [59,81] or the bactericidal/permeability-increasing protein [80].

The studies were complemented by a comparison of LPS and GSL with respect to physico-chemical properties such as the fluidity of pure glycolipid-membrane systems, the molecular charge and the molecular area of the glycolipid molecules arranged in monolayers at the air-water interface. Due to experimental restrictions, most of the investigations were, however, restricted to deep rough mutant LPS (Re LPS) and to the GSL with the shortest sugar moiety, GSL-1. In our previous work mainly Re LPS from *Salmonella enterica* sv Minnesota strain R595 (R595 LPS) was used and later also that from *Escherichia* coli strain F515 (F515 LPS). These two LPS differ in the degree of acylation—R595 LPS carries a seventh fatty acid in non-stoichiometric substitution—and substitution by aminoarabinose and phosphoethanolamine at the phosphates [53].

Physico-chemical characteristics

Phase behaviour: The characteristics of a biological membrane as a permeation barrier depend, among other properties, on the fluidity (state of order) of the hydrocarbon chains of the constituting lipid molecules. Furthermore, a relatively high fluidity (low state of order) is a prerequisite for the preparation of stable asymmetric bilayers. The state of order of membrane lipids can be determined either *via* fluorescence polarization spectroscopy using the fluorophore DPH or *via* Fourier transform-infrared (FT-IR) spectroscopy. In the latter case, the peak position of the symmetric stretching vibration of the methylene groups $\nu_s(CH_2)$ can be taken as a measure of the state of order of the acyl chains. Deep rough mutant Re LPS from *S. minnesota* R595 and from *E. coli* F515 undergoes a sharp gel↔liquid ($\beta \leftrightarrow \alpha$) phase transition of the hydrocarbon chains at $T_c \approx 32°C$ [11], whereas GSL-1 shows a continuously decreasing state of order in the temperature range from 15 to 60°C [79]. Most interestingly, however, the respective states of order for Re LPS and GSL-1 are nearly identical at the physiological temperature of 37°C. Both glycolipids have a significantly higher state of order at 37°C than the phospholipid mixture of the inner leaflet [79].

Molecular area: The molecular area of lipid molecules can be calculated from the area of a lipid monolayer at the air-water interface composed of a known number of molecules and compressed to a lateral pressure of 30 mN m^{-1}, which is assumed to resemble the lateral pressure in a bilayer membrane under physiological conditions [9,18,35,47]. The molecular areas thus determined are 1.3 nm^2 for Re LPS from *E. coli* and 0.5 nm^2 for GSL-1, respectively [79].

These data are important for the calculation of the respective surface charge densities of monolayers made from Re LPS or GSL-1 (see next section).

Membrane potential

Electrostatic properties of membranes are, in addition to their hydrophobic characteristics, of particular importance for interaction with biomolecules and may play a crucial role in many membrane-associated biological effects. In lipid bilayers, several distinct electrostatic potentials arise from different sources and superimpose to a characteristic profile of the intrinsic membrane potential. The various potentials are summarized briefly below before a discussion on the particular contribution of glycolipids.

Charged components of lipid molecules (such as phosphate, carboxyl, or amino groups) expressed at the membrane surface generate a surface potential, which—according to Gouy and Chapman (for a review see [14])—decreases exponentially within a distance of a few nanometers from the membrane surface. According to the specific lipid composition of the membrane, surface potentials of biomembranes are typically negative and of the order of a few tens of mV. Nevertheless, they play an important role in controlling biological processes within the immediate vicinity of both bacterial and host cell membranes (eg, repulsion of anionic or attraction and binding of polycationic compounds causing, in turn, alterations of the potential [5]).

The largest contribution to the intrinsic membrane potential arises from the dipole potential (U_D) inside the lipid bilayer ($\leq$ +300 mV). Experimental data as well as electrostatic calculations allow the conclusion that this potential is caused by oriented dipoles of bound water molecules, in particular in the interface region [90]. Unlike the surface potential, the internal dipole potential is independent of the ionic strength of the electrolyte solutions on both sides of the membrane. The dipole potential is responsible for observed differences in the passive permeability of cationic and anionic molecules through the membrane and for conformational changes in proteins when they are incorporated into the membrane [13,20]. An additional energy barrier arises from the Born self-energy (Born-potential, U_B), which is the energy necessary to transfer a charge from a medium with a high dielectric constant $\in$ (water) to one with a low $\in$ (membrane) [56].

Resulting from differences in the surface potential as well as in the dipole potential, lipid asymmetry may provoke a potential difference between the two surfaces of the bilayer membrane. An extreme asymmetry in charge den-

sities as well as in headgroup conformation occurs for asymmetric LPS/phospholipid bilayers representing the outer membrane of typical Gram-negative bacteria. The phospholipid mixture (PL) of the inner leaflet consists in the case of *S. enterica* sv *typhimurium* of a mixture of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG) in a molar ratio of PE : PG : DPG = 81 : 17 : 2 [50]. At neutral pH, of the major constituents only the PG molecules carry negative charges (one per molecule). Most simply, the LPS component of the membrane consists of Re chemotype-like molecules. In this case, each molecule carries four negative charges (referred to F515 LPS). For the calculation of the surface charge densities of each leaflet, ie, the number of charges per unit area, the molecular area occupied by the respective lipid molecules has to be considered (see above), which is larger by a factor of four for F515 LPS (1.3 nm^2) than for phospholipids (0.6 nm^2) [81]. Thus, the surface charge density of the F515 LPS leaflet (–3.1 elementary charges nm^{-2}) is by a factor of ten higher than that of the PL leaflet (–0.2 elementary charges nm^{-2}). At pH 7, the GSL-1 molecule carries only 0.5 negative charges per molecule, as determined from FT-IR-measurements of the pK-value. As the molecular area is only 0.5 nm^2, the surface charge density is –1.0 elementary charges nm^{-2} [81]. The height of the surface potential is one determinant for the cell function as well as for the interaction of drugs with the membrane. It can be calculated from the above surface charge densities according to the Gouy equation [37]:

$$U_{GC} = \frac{2kT}{Ze_0} asinh \frac{\sigma}{(8 \in \in_0 kTN_A c)^{1/2}} \quad (1)$$

where k is the Boltzmann constant, T the absolute temperature, $\in$ the relative dielectric number of the bathing solution, $\in_0$ the dielectric constant, e_0 the elementary charge, Z the valence of the counterions, c the concentration of the bathing solution, N_A Avogadro's number, and σ the surface charge density (all to be taken in SI units). Instead of the concentration, frequently the ionic strength is used. The thus calculated values for the GSL-1, F515 LPS, and the PL layers are given in Table 1.

Further determinants are the height and the profile (innermembrane potential difference $\Delta\Phi$) of the potential wall, which can be determined experimentally [56]. To this end, the I/U-characteristics of the bilayers can be measured for bilayers doped with the K^+-carrier nonactin. The evaluation of the I/U-curves is done according to procedures described previously [62]. Briefly, the current I as function of the voltage U applied with the voltage clamp is given by Schoch *et al* [56]

$$I_m = K\frac{(\Delta\Phi + (n_2 - n_1)U)}{(n_2 - n_1)} \cdot \frac{e^{a\,U} - 1}{e^{\alpha\cdot(\Delta\Phi + n_1 U)} - e^{\,a\,n_2 U}} \quad (2)$$

where $a = (Z \cdot e_0)/(k \cdot T)$, K is a constant for each membrane (depending among others on its area and thickness), n_1, n_2 are the edges of the potential walls for the two leaflets, and $\Delta\Phi$ the potential difference between these edges. The three parameters describe the shape of the trapezoidal energy barrier and are determined from the experimental curves by computer fitting of the above equation.

A comparison of Re LPS/PL and GSL-1/PL membranes has been given in [79]. The respective intrinsic membrane potential profiles are plotted in Figure 1, reflecting the differences in the respective Gouy-Chapman potentials (Table 1) and in the sign as well as in the absolute height of the potential difference $\Delta\Phi$ of the glycolipid leaflets. As the PL side is left unchanged in both systems, the contribution of the glycolipid side to the dipole potential is much smaller in the case of GSL-1 than in the case of F515 LPS. The effects of the differences in the potential profile, especially of the surface potential on the function of the outer membrane are discussed in the following sections for porin function and the interaction of PMB with the outer membrane.

Porin function

The outer membrane of Gram-negative bacteria serves as a molecular sieve allowing small hydrophilic molecules (≤600 Da) to permeate through particular pores that are formed by special outer membrane proteins (Omp) termed porins [48]. The porins, in general, have characteristic molecular weights between 30 and 50 kDa and normally form trimers within the outer membrane [2,36].

Although porins have been studied intensively for many years (for reviews see [4,28,55]), there still exist controversial aspects on some fundamental questions. To these belong, among others, the influence of LPS on protein biosynthesis and on functional properties such as channel size, gating behavior, oriented incorporation, and the orientation of porins in the membrane.

Contradictory results on the degree of voltage sensitivity of bacterial porins (voltage gating) have been reported even for the same porin, but investigated in different systems. This holds in particular for OmpF [34,43] and OmpC [12,33] of various strains of *E. coli*. Possible reasons for these discrepancies might be found in the different mem-

Table 1 Molecular area, molecular charge, and surface potential according to the Gouy equation for GSL-1, F515 LPS, and PL calculated for a bathing solution containing 50 mM KCl and 5 mM $MgCl_2$, 5 mM HEPES (resulting ionic strength of the bathing solution: 65 mM); pH 7, T = 37°C

Lipid	Area/ molecule, nm^2	Charge/ molecule, e_0	Surface charge density e_0 nm^{-2}	Surface potential, mV
F515 LPS	1.3	–4.0	–3.1	–187
GSL-1	0.5	–0.5	–1.0	–128
PL	0.6	–0.2	–0.3	–72

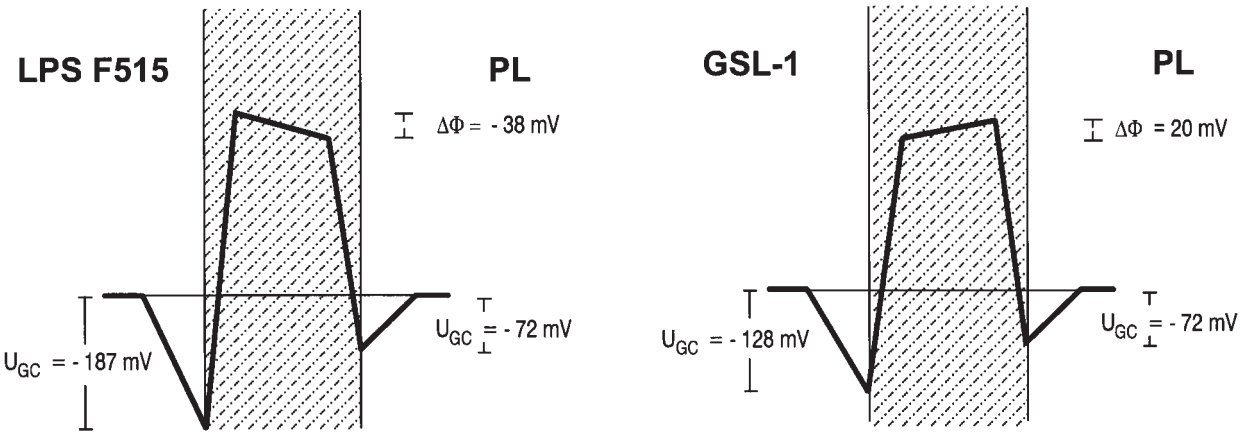

Figure 1 Intrinsic membrane potential profiles for asymmetric F515 LPS/PL (left) and GSL-1/PL (right) bilayers. The Gouy-Chapman potentials were obtained from Equation 1 and the trapezoidal inner membrane potential differences, $\Delta\Phi$, were calculated from the respective fits of Equation 2 to the experimental data (adapted from [79] with permission of the Biophysical Society).

brane systems used for reconstitution, salt concentrations, pH, and, maybe most importantly, the presence or absence of LPS. For example, Lakey and Pattus [34] compared the voltage-dependent gating of OmpF from *E. coli* reconstituted into symmetric lipid membranes prepared as black lipid membranes (BLM) or according to the Montal–Mueller and Schindler techniques, respectively. Independent of the method chosen, the authors observed channel closing of the incorporated pores; however, the absolute height of the gating voltage differed for the different techniques.

In 1989, workers in our laboratory, applying the Montal–Mueller technique, for the first time successfully reconstituted the lipid matrix of the outer membrane as an asymmetric LPS/PL bilayer [61]. Using this reconstitution model, we were then able to answer questions on the influence of the lipid environment, in particular that of the glycolipid, on the function of porin channels. For these experiments, we used LPS-free porins from *Paracoccus denitrificans* ATCC 13543, which we incorporated into different symmetric and asymmetric phospholipid and LPS/PL and GSL/PL planar bilayers [78,79].

In accordance with studies of Parr *et al* [52] we were able to show that the presence of LPS is not required for manifestations of channel activity. Therefore, porin incorporation was also observed in asymmetric GSL-1/PL and even in pure phospholipid membranes. LPS and GSL, however, influence other parameters of porin function such as their incorporation rate into the reconstituted membrane and their gating behavior, ie, transmembrane potential-dependent channel closing was observed. The incorporation rates into pure phospholipid bilayer systems were more than a factor of ten lower than into asymmetric membranes with an Re LPS or GSL-1 leaflet on the opposite side. Porin channels, however, did not incorporate at all when added to the glycolipid side. The differences in the incorporation rates for the different membrane systems cannot be accounted for by differences in the surface charge densities between the two membrane leaflets of each system, because no differences in the incorporation rates into PG/PL and PL/PL bilayers were observed. This implies the existence of specific molecular interactions pulling the porin molecules into the bilayer when they reach the glycolipid leaflet. This observation is, therefore, a strong indication that specific interactions between the porin molecules and the glycolipids take place within the glycolipid leaflet and is in accordance with the model of Weiss *et al* [75,76], who propose that the very polar zones on the outer surfaces of the barrel-shaped porin monomers with their numerous negatively charged residues are directed towards the extracellular medium. According to these authors, the carboxylate groups of the porins are likely to participate in the strong and tight network of divalent cations and LPS carboxylate groups in such a way that the interface between LPS and porin would become as tight as the LPS layer itself. These interactions seem also to be important in the interaction of the porin with GSL-1.

In vitro studies concerning the influence of LPS and GSL on the folding of PhoE, an outer membrane porin from *E. coli*, into its folded monomeric native-like state, however, showed that GSL-1 was less effective than LPS [17]. In the same study, it was shown that a three-dimensional nonlamellar aggregate structure of the lipid A part of LPS enhances the folding efficiency. The lipid A part of the LPS of *P. denitrificans*, however, adopts a lamellar aggregate structure—as does GSL-1—and has a reduced folding efficiency. Therefore, it may be assumed that for the porin of *P. denitrificans*, which was used for studies of the bilayer membrane the aggregate structure is not so important as in the case of PhoE, thus leading to comparable incorporation rates into GSL-1/PL and Re LPS/PL membranes.

The complete prevention of porin incorporation from the glycolipid side may be explained by the rigidity of the corresponding membrane leaflets—both glycolipids have, at physiological temperature a higher state of order than most natural phospholipids—or the steric hindrance caused by the sugar moieties of the glycolipids.

Using this experimental model we were able to study gating effects with respect to bilayer asymmetry, in particular with respect to the resulting charge distribution and the arising potential gradient in the case of Re LPS/PL and GSL-1/PL membranes [78,79]. We have observed voltage-

dependent closing of porin channels in symmetric as well as in asymmetric bilayers [in the current/voltage curves (Figure 2) the linear current increase stops when a threshold voltage, the gating voltage, is exceeded]. This gating voltage, however, depends on the lipid composition of the membranes. For asymmetric membrane systems, the absolute value of the gating voltage was lower when the applied potential was negative on the side of the more negatively charged lipids. Taking into account the surface charges and Gouy-Chapman potentials derived therefrom, a correlation between the height of the surface potential—which is most negative in the case of LPS—on the one hand and the gating voltage on the other could be stated. The importance of the glycolipids in forming the outer layer of the outer membrane for porin gating is further supported by the recent observation that the extracellular domains of OmpF are involved in channel closing [45]. These data emphasize the strong influence of the lipid composition on the voltage-dependent gating of porin channels.

Interaction of polymyxin B with the outer membrane

As a constituent of the outer leaflet of the outer membrane, GSL influences the susceptibility of *S. paucimobilis* (eg strain IAM 12576) towards membrane-active antibiotics such as polymyxin B (PMB). PMB consists of a pentacationic amphipathic lipopeptide characterized by a heptapeptide ring linked to a peptide side chain, which terminates with a short fatty acid residue [19,64]. PMB has a molecular weight of about 1200 Da and thus, is inhibited from diffusing through the porin channels. It has rather to penetrate the lipid matrix of the outer membrane. In this context, the self-promoted uptake has been discussed [25]. The bactericidal action of PMB can be divided into two major steps, binding to and permeabilization of the outer membrane and induction of lethal leakages in the cytoplasmic membrane [71]. Especially for the first step, the lipid assembly of the outer membrane is of importance.

Whereas Gram-negative bacteria, in general, are sensitive towards the antibacterial action of PMB, *S. paucimobilis* and LPS-carrying species like *Proteus mirabilis* belong to the *a priori* resistant species. It is known that the 4′-phosphate of the lipid A and the first Kdo of the LPS of the PMB-resistant strain *P. mirabilis* R45 (R45 LPS) is substituted by cationic L-Ara*p*4N [29]. Also, it has been shown that in PMB-resistant mutants of *E. coli* (strains SC 9252 and SC 9253), *S. enterica* sv *typhimurium* LT2, and *S. marcescens* 111, L-Ara*p*4N and 2-aminoethanol were present in the lipid A, whereas these substituents were absent in the PMB-sensitive strains [49,60,70]. These substitutions result in a reduction of the molecular charge and surface charge density (changes in the molecular area can be neglected). Also for GSL-1, a reduced surface charge density can be observed compared to Re LPS [81] (see above).

In early work on the membrane interactions of PMB, contradictory results were obtained. Whereas Antonov *et al* [1] proposed a carrier mechanism for the action of PMB, Miller *et al* [40] who investigated the interaction of PMB with BLM made from various phospholipids differing in their surface charge density found that PMB caused no conductance increase but led to an unspecific destabilization of the membranes [40]. In contrast to this early work, we observed that addition of PMB to asymmetric glycolipid/phospholipid membranes leads to the formation of transient membrane lesions [59,81] (Figure 3). Furthermore, in our investigations we pointed out the role of negative charges for the size of PMB-induced membrane lesions, and for the PMB concentration needed to induce

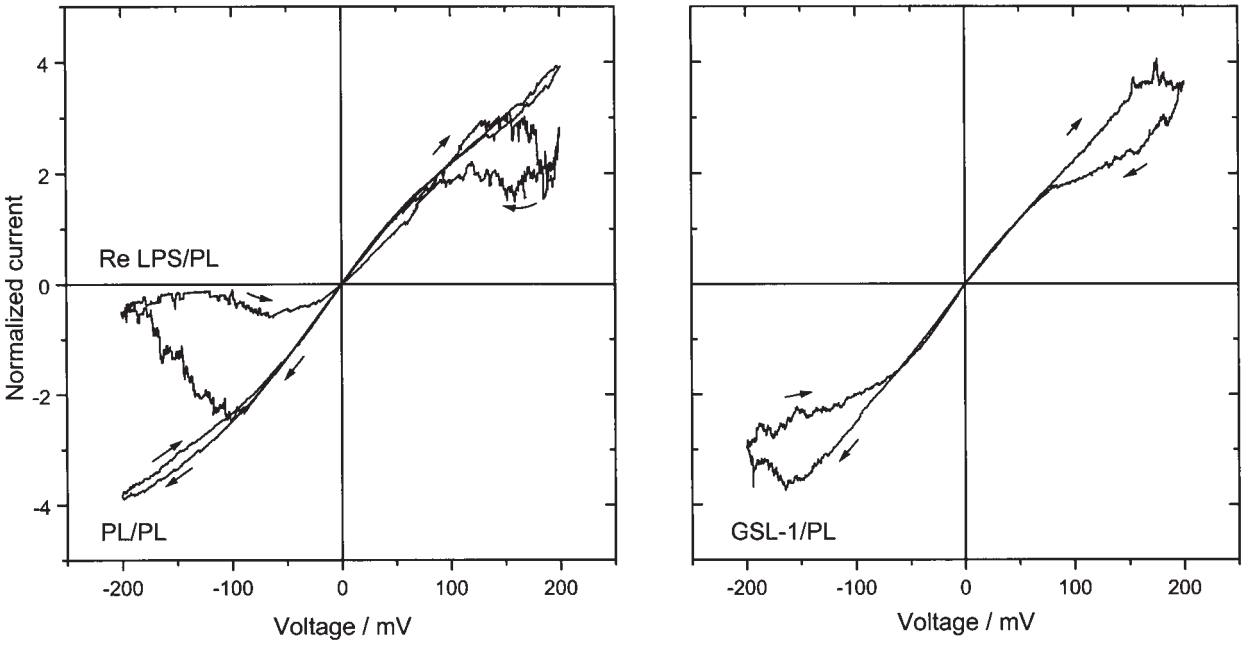

Figure 2 Current/voltage curves for various membranes doped with the porin from *P. denitrificans* (porin is added to the second-named side). Current is normalized to the value at 40 mV (ie, when all channels are open). Bathing solution: 100 mM KCl, 10 mM $MgCl_2$, 5 mM HEPES, pH 7, T = 37°C (data taken from [78] and [79] with permission of Elsevier Science BV and the Biophysical Society, respectively).

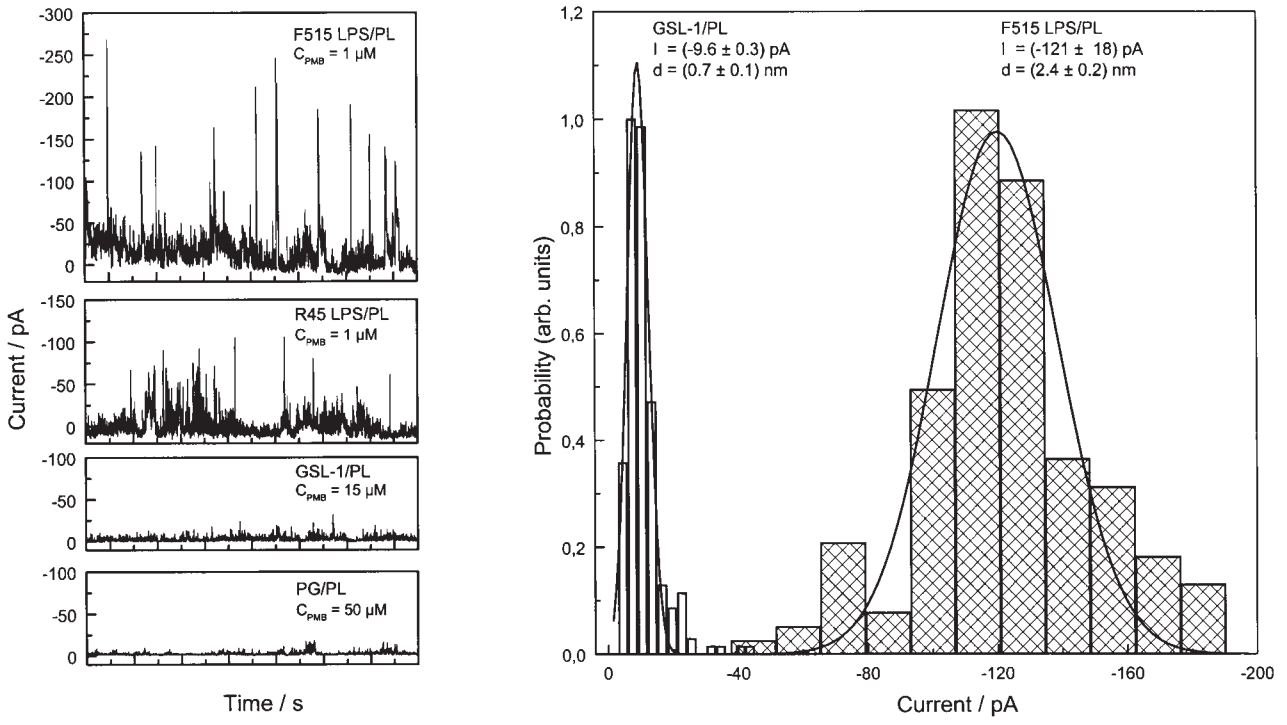

Figure 3 Current fluctuations (left) in dependence on time in the initial phase after the addition of various concentrations of PMB to asymmetric bilayers (the side of PMB addition is named first). Distributions of the amplitudes of current fluctuations induced by addition of PMB to the glycolipid side of F515 LPS/PL and GSL-1/PL bilayers (right), fitted by Gaussian distributions (solid curves). Clamp voltage of –80 mV (side of PMB addition grounded), bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7, T = 37°C (adapted from [81] with permission of Springer-Verlag).

these fluctuations [81] by investigating the interaction of PMB with various differently composed bilayer membranes, including those resembling the lipid matrices of the outer membranes of various species of Gram-negative bacteria (Figure 3, left).

The addition of PMB at a final concentration of 1 μM to the LPS side of an asymmetric F515 LPS/PL planar bilayer membrane, which is in the range of the minimal inhibitory concentration [64], and a clamp voltage of –20 mV (LPS side grounded), led to a noise-like increase in the macroscopic current with time, which terminated in a plateau-like steady state typically within a few minutes after peptide addition. Microscopically, the initial phase of the interaction was characterized by short transient current fluctuations, which are indicative for the induction of transient membrane lesions (Figure 3, left).

A comparison of the lesion-inducing capability of PMB added to the glycolipid side of different asymmetric glycolipid/PL membranes clearly showed that the PMB threshold concentration and the threshold voltage needed to induce current fluctuations as well as the size of the PMB-induced membrane lesions are correlated with the surface charge density of the glycolipid layer. Thus, the conductivity responses after PMB addition (concentration of 1 μM, clamp voltage of –20 mV) were very similar for F515 LPS and R595 LPS [59] but significantly different for the other glycolipids. This should not be surprising in view of the fact that the two LPS have nearly identical structures and differ only slightly in their effective molecular charge. Under the same conditions, the single-channel fluctuations did not occur for GSL-1/PL bilayers and were considerably smaller and appeared less frequently for R45 LPS/PL membranes. For GSL-1/PL membranes, at PMB concentrations of 15 μM, current fluctuations were observed, which were, however, very small.

As an example, in Figure 3, right, the distributions of the single channel currents at –80 mV and the resulting average single channel conductivities and diameters of the underlying membrane lesions are compared for GSL-1/PL and F515 LPS/PL membranes. The mean diameters, d, of a single membrane lesion could be calculated from the mean current, I, of the single fluctuations at a clamp voltage, U, in a bathing solution of the specific conductance, σ, according to the relation $I = (\pi \cdot \sigma \cdot d^2 \cdot U) / (4 \cdot l)$. For this, a circular geometry of the lesion and a membrane thickness, l, of 6 mm was assumed. Thus, the diameter of the lesions is matrix-dependent and only 0.7 nm in the case of GSL-1, but about 2.4 nm in the case of R595 LPS and F515 LPS. Teuber and Bader [66] described a correlation of the binding capacity of PMB and the surface charge of isolated cytoplasmic and outer membranes from *S. typhimurium* G30. Beyond this, we could demonstrate a good correlation between the diameter of the lesions and the molecular charge and the surface charge density, but not the molecular area.

In the context of the self-promoted uptake, the PMB-induced transient membrane lesions attain importance as pathways for facilitated diffusion. A prerequisite for a facilitated diffusion of PMB through the lesions—which are transient water-filled pores—is, of course, a minimal diameter large enough to allow single PMB molecules to pass. In the cases of F515 LPS/PL and R595

LPS/PL membranes, this prerequisite is fulfilled. The diameters of the lesions are about 2.4 nm, whereas the cross-section of the PMB molecule is 1.23 nm [19] and the respective bacteria are PMB-sensitive. The diameters of the lesions in the other lipid matrices relevant for Gram-negative species are 1 nm for R45 LPS/PL or 0.7 nm for GSL-1/PL membranes. The respective Gram-negative species are PMB-resistant [29,85].

Our findings provide an explanation for the influence of the substituents on the expression of PMB resistance: the reduction in surface charge density by the cationic substitutions by L-Ara*p*4N and 2-aminoethanol leads to a reduction in the number of bound PMB molecules and to a reduction in the size of the transient lesions. The importance of the molecular charge for PMB activity is further emphasized by the data on GSL-1/PL membranes. The GSL-1 molecule carries *per se* only one half effective negative charge, and the PMB-induced lesions are extremely small.

Because addition of detergents like SDS at amounts close to their critical micellar concentration (CMC) also induces transient membrane lesions, and light scattering experiments show that PMB forms aggregates, not necessarily micelles, we have proposed a detergent-like action for PMB [59,81]. According to this model, electrostatic attraction causes an accumulation of positively charged PMB molecules at negatively charged membrane surfaces dependent on the surface charge density to a concentration higher than the CMC, finally leading to formation of PMB aggregates. The insertion of these PMB aggregates, which is enhanced by electrostatic interaction, leads to formation of locally restricted lipid-PMB complexes of different stoichiometries depending on the molecular charge of the lipids. These complexes exert a local stress in the lamellar bilayer, which finally causes the formation of membrane lesions. The lesions are larger the more PMB molecules are bound per lipid molecule. Thus, a threshold stoichiometry must be overcome to allow self-promoted uptake resulting in bacterial death. This condition is not fulfilled in the case of the outer membrane of *S. paucimobilis*.

The limited lifetimes of the induced lesions may be explained by the lateral diffusion of PMB molecules in the membrane due to concentration gradients leading to a decrease in the local PMB concentration below the critical value required for formation of nonlamellar membrane disturbances. Also, the macroscopic steady state can be explained by lateral diffusion leading to an equilibrium concentration of PMB in the membrane. Thus, further addition of PMB after the steady state is reached does not lead to a further increase in membrane current [59].

With these data it could be shown that the replacement of LPS by GSL in the outer leaflet of the outer membrane is the reason for PMB resistance of *S. paucimobilis*.

Activation of the complement system by LPS and GSL surfaces

In this section, we compare the role of LPS and GSL as constituents of the outer layer of the outer membrane as activator substances for the C system. The C system is an important part of the host defense against invading bacteria. It consists of at least 20 serum proteins and plays an important role in the early host defense against invading bacteria [42]. Its activation can lead to direct lysis of bacteria *via* the formation of transmembrane pores built up by the alignment of C9 monomers [7,8,69]. The C system can be activated along at least two distinct pathways, the classical and the alternative pathways. Both pathways can be initiated with or without the participation of antibodies [32]. Gram-negative bacteria activate C mainly by the surface glycolipids anchored to the outer leaflet of the outer membrane [16], and the C cascade directs its lytic activity against the surfaces of invading bacteria [6]. In particular, the formation of the membrane attack complex, which finally leads to cell lysis, is catalyzed by the bacterial surface.

The activation of the classical pathway by dispersed endotoxin aggregates was found to depend on the physicochemical properties of the applied preparations such as the aggregate size of the lipids [21,23,51,83]. Therefore, study of C activation requires comparability of the physicochemical properties of the various activating surfaces. This requirement is fullfilled when planar membrane reconstitution models are used. To study the mechanisms of complement activation by invading Gram-negative bacteria, in particular with the aim of defining functional groups of the surface glycolipids directly involved in the activation process, utilization of asymmetric glycolipid/phospholipid membranes should be a method of choice. In a planar bilayer membrane, the reconstituted surface glycolipids are presented in the natural orientation independent of their primary chemical structure. This way, an influence of the state of lipopolysaccharide aggregation on complement activation, which has been described by Wilson and Morrison [83], can be ruled out. In the literature, few investigations into conductance changes induced by complement as measured in symmetric planar phospholipid bilayers [3,39,63,84,88] as well as in patch-clamped cells [27] have been reported. The results of these studies concerning pore size, pore stability, and also the requirements of various complement components for induction of conductance changes differ considerably. To our knowledge, the only investigations using asymmetric LPS/PL or GSL/PL membranes have been done in our laboratory.

In many of the early investigations, the complement system has been activated by addition of C5b-6 (reactive lysis) [3,63,88], after addition of antigen and antibody [44,84], or adsorption of antibodies onto the membranes [27]. In all cases, an increase in membrane conductivity was observed. From the increase in conductivity in single steps and bursts or from the amplitude of current fluctuations, estimates for the diameter of the induced membrane pores/lesions, based on the assumption of cylindrical water-filled channels, was derived. These calculations were based on two different pore lengths: a pore length of 6 nm according to the membrane thickness or a length of about 15 nm based on electron microscopic observations [67,68]. Assuming a pore length of 15 nm, two different pore sizes have been found: small pores about 1–2 nm in diameter with short lifetimes and leading to current fluctuations [88] and larger ones about 8–10 nm in diameter, which were more stable [3]. Whereas the smaller fluctuations were suggested to represent transient precursors of the final membrane attack

complex [27], the larger ones were correlated with the insertion of poly C9 complexes. In all these studies, the influence of LPS on complement activation, especially on the activation pathway and the role of the lipid matrix for the pore size could not be accurately determined.

Concerning complement activation by isolated LPS and lipid A as it may depend on the length of the polysaccharide chain, fundamental work has been published by Vukajlovich *et al* [72–74]. These authors found that both, LPS and lipid A, led to an antibody-independent activation of the complement cascade, but the pathway of activation depended on the length of the polysaccharide chain: lipid A and Re LPS activated the complement system *via* the classical pathway, whereas lipopolysaccharides with a longer polysaccharide chain (R-mutant chemotype LPS beyond Re LPS) activated *via* the alternative pathway. Mey *et al* [38] showed that the acylation of lipid A of a *Klebsiella pneumoniae* I-145 LPS was important for activation of the alternative pathway of the complement system, and Clas *et al* [15] pointed out the importance of the length of the sugar chains for binding of C1q. The disadvantage of these studies is the fact that LPS or lipid A were presented as aggregates in an aqeuous environment rather than in their natural structural arrangement, ie as constituent of a lipid bilayer.

Our reconstitution model of the lipid matrix of the outer membrane [61] has allowed us to compare C activation induced by LPS and GSL with respect to the activation pathway as well as to the pore size [46,58,79] within a microbial-like environment. Whereas no changes in conductance were detected when serum was added to the PL side of the membrane, serum addition to the Re LPS side led to a significant increase in membrane conductivity. At the beginning of the conductance trace, the incorporation of C9 monomers were reflected by a stepwise increase of conductance—about nine steps—until the formation of one individual pore was completed and the assembly of the next pore began. The small current steps either had identical amplitudes or exhibited a quadratic increase in the amplitudes. From these current traces, a model for the formation of complement pores could be derived, which is based on the subsequent incorporation of about nine C9 monomers to form either a cylindrical pore (corresponding to the quadratic increase in conductance) or a so-called leaky patch, which is only on one side aligned by C9 monomers (corresponding to current steps of the same amplitude). From the amplitude of the current increase related to the formation of one complement pore, a pore diameter of about 8 nm was determined [58]. Experiments with LPS differing in the polysaccharide moiety (Re, Rd_1,Rd_2, and Rc) and GSL-1 [46,79] led to similar results.

In studies aiming at elucidation of pathways of complement activation, we found that neither the Ca^{2+} chelator EGTA nor the depletion of C1q had an influence on the activation by GSL-1 (Figure 4a), whereas in the case of lipid A, Re LPS (Figure 4b), and LPS Rd_2 a significant deactivation was observed. Furthermore, serum from which factor B was depleted (BdepHS) showed no activity on GSL-1/PL bilayers. Activity could be restored by addition of physiological concentrations of purified factor B (Figure 4a). From these results it is concluded that the investigated LPS activate the classical pathway, whereas GSL-1 activates the alternative pathway. Activation of the classical pathway of LPS was antibody independent [46,58]. In our studies [46,79], alternative pathway activation and the first steps of membrane attack occur even at low serum concentrations (dilution 1:500), whereas the lysis of *E. coli* K12 W1485 was completely inhibited by serum dilution by a factor of 16 or more [57]. Our finding that Rd_2 LPS activates the classical pathway is in contradiction to previous work of Vukajlovich *et al* [74] who found that all LPS structures with a sugar moiety longer than that of Re chemotype LPS activate the alternative pathway. The additional L-*glycero*-D-*manno*-heptose in Rd_2 chemotype

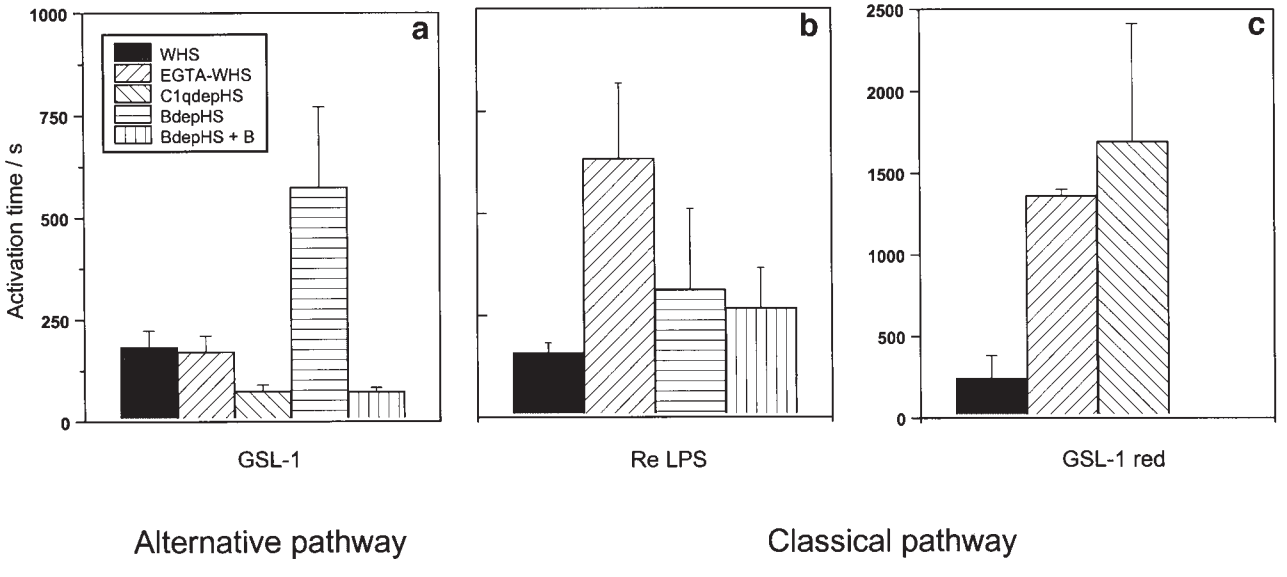

Figure 4 Activation times for different sera on the GSL side of GSL-1/PL (a), Re LPS (b) and GSL-1 red/PL bilayer (c). Serum concentration: 0.3% Re LPS, and 0.2% GSL-1 red. Bathing solution: 100 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.4, T = 37°C (adapted from [46] with permission of Springer-Verlag).

LPS was interpreted by these authors to have a critical recognition role for activation of the alternative pathway and a downregulation of the classical pathway activation. For an understanding of this discrepancy, it has to be considered that in the studies of Vukajlovich and coworkers [74], LPS was applied in aggregated form in aqueous buffer and with the spatial arrangement of the target structure for the C proteins being poorly defined. Also Brade *et al* [10] found that the physico-chemical state and the environment of lipid A modulated its antigenicity. Using liposome-incorporated instead of aggregated antigens as inhibitors in the passive-hemolysis inhibition assay, they were able to show that nonspecific reactions were avoided and specific ones were enhanced. Furthermore, Tenner *et al* [65] showed that whole *E. coli* J5 cells, which express an Rc chemotype LPS on their surface, activate C1 and with that the classical pathway.

Alternative pathway activation by GSL-1 is surprising in the light of the situation observed for endotoxin preparations. The reported [74] activation of the alternative pathway was found for LPS with longer sugar chains, whereas GSL-1 contains only one sugar. Also gangliosides with five or more monosaccharides activated the alternative C pathway [51]. This behavior suggests, in agreement with Grossman and Leive [24], that the chemical character of the sugar rather than the length of the sugar moiety determines the activation pathway. This assumption is strengthened by the observation that membranes made from a reduced GSL-1 derivative (GSL red) carrying a glucose instead of the glucuronic acid, activate the C system via the classical pathway [46] (Figure 4c). It further suggests that not the presence of a negatively charged group but rather the chemical nature of the substituent determines the pathway of C activation.

The data reviewed in this contribution show that GSL-1 and Re LPS, despite differences in detail, exhibit a high degree of similarity in the investigated parameters and properties, and it may thus be concluded that these two glycolipids are very similar with respect to their function as antigenic surface structures as well as to their contribution to the function of the outer membrane of the respective Gram-negative species as a diffusion barrier.

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