J. Membrane Biol. 109, 95–103 (1989)

The Journal of
Membrane Biology

Reconstitution of the Lipid Matrix of the Outer Membrane of Gram-Negative Bacteria as Asymmetric Planar Bilayer

Ulrich Seydel, Guido Schröder, and Klaus Brandenburg
Forschungsinstitut Borstel, Division of Biophysics, D-2061 Borstel, Federal Republic of Germany

Summary. This paper is a report on the reconstitution of the lipid matrix of the outer membrane of Gram-negative bacteria as an asymmetric planar bilayer. This is the first time that a planar membrane is described, which consists on one side of a phospholipid (PL) mixture and on the other side of lipopolysaccharide (LPS). Therefore, strong emphasis is placed on a physical characterization of this membrane via its electrical properties. The membranes were prepared from spread monolayers or from vesicle-derived monolayers. Contrary to observations for symmetric phospholipid membranes, specific capacitances of (0.67 ± 0.02) $\mu F \cdot cm^{-2}$, breakdown voltages between 200 and 400 mV and specific conductances between 10^{-8} and 2×10^{-7} $S \cdot cm^{-2}$ were obtained independent of the preparation method. The LPS-containing membranes were stable up to 3 hr if they were formed and kept at temperatures above the hydrocarbon chain melting temperature of the LPS. For the specific capacitance, a dependence on the aperture radius was observed. This is explained by assuming a toroidal transition zone at the rim of the aperture.

First results on the action of the pore-forming α-toxin from *Staphylococcus aureus* on bilayers of different composition demonstrate particular characteristics of this asymmetric bilayer system. The pore-formation rate is highest in symmetric phospholipid bilayers, considerably lower in asymmetric PL/LPS systems and fully inhibited in LPS/LPS systems.

Key Words outer membrane · planar lipid bilayer · lipopolysaccharide · α-toxin · membrane reconstitution

Introduction

The cell envelope of Gram-negative bacteria contains beside the cytoplasmic membrane and the peptidoglycan layer, common also to Gram-positive bacteria, an additional barrier, the outer membrane, which is located outside the peptidoglycan layer. Regarding the lipid components, this outer membrane is extremely asymmetric. Whereas the inner leaflet contains only phospholipids (PL), the outer leaflet is built up mainly from lipopolysaccharides (LPS) consisting of a polysaccharide moiety and a covalently linked lipid component termed lipid A, anchoring the LPS in the outer membrane (for review *see* Rietschel et al., 1984).

For the bacterial cell, the outer membrane represents a permeation barrier giving protection to host defensive factors and also various antibiotics, which are known to be effective against Gram-positive bacteria (Nikaido, 1979; El-Falaha, Russel & Furr, 1983; Nikaido & Vaara, 1985). Because the organization, stability and barrier function of the outer membrane seems to be essential for the integrity, growth and the survival of bacterial cells (Rietschel et al., 1987), it is clearly important to elucidate the transport properties of this membrane and its susceptibility against host defensive factors as well as drugs.

The investigation of these functions of the outer membrane is complicated by its complexity, therefore the reconstitution of simpler model systems—e.g. in a first step the unmodified lipid matrix—are helpful to study the function of single components of the outer membrane, particularly LPS. The influence of other constituents like proteins may, in a second step, be studied by their subsequent introduction.

Since the vesicle systems frequently used for transport measurements do not allow the simulation of the asymmetric lipid distribution of the outer membrane, we have reconstituted the outer membrane lipid matrix as an asymmetric planar bilayer system on the basis of the Montal-Mueller technique (Montal & Mueller, 1972), in which the bilayer is prepared from monolayers spread from nonpolar solutions of the lipids, and of the Schindler technique (Schindler, 1980), which is based on the spontaneous formation of monolayers from a vesicle solution. This way a possible influence of solvents on the bilayer properties of our particular system could be studied.

This paper reports on the reconstitution of the

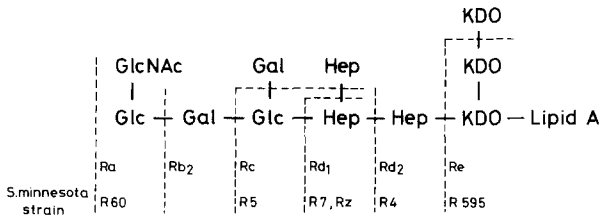

Fig. 1. Schematic structure of the core region of various lipopolysaccharides from rough mutants of *Salmonella minnesota*. The wild-type (S form) LPS additionally contains the covalently linked O-chain. *Re* to *Ra*, in accordance with general terminology, refer to the different rough mutant LPS, and R595 to R60 are the respective strains of *S. minnesota*. Abbreviations: *KDO* = 2-keto-3-deoxyoctonate, *Hep* = heptose, *Glc* = glucose, *Gal* = galactose, *GlcNAc* = glucosamine-acetyl

lipid matrix of the outer membrane of Gram-negative bacteria as an asymmetric planar bilayer and on its characterization via the measurement of its electrical properties.

First experiments were performed on the particular behavior of this asymmetric bilayer system in the interaction with a pore-forming protein, the α-toxin from *Staphylococcus aureus*, in comparison to phospholipid membranes. The results from these experiments demonstrate the importance of the bilayer lipid composition for the respective action and function of proteins on or in biological membranes.

Materials and Methods

Lipids and Other Chemicals

As LPS that of *Salmonella minnesota* was used. In Fig. 1 the structure of various rough mutant LPS from this species is shown. They consist of the hydrophilic core oligo-saccharide portion and a covalently linked lipid moiety, termed lipid A (Lüderitz et al., 1982).

The lipid A of *S. minnesota* (Fig. 2) is composed of a β-D-glucosaminyl-(1-6)-α-D-glucosamine disaccharide, which carries two phosphoryl groups in positions 1 and 4'. This hydrophilic back-bone is acylated by six or seven fatty acid residues either ester or amide linked. The phosphoryl groups may be substituted by phosphoethanolamine and 4-amino-arabinose.

In our experiments mainly the LPS of the deep rough mutant R595 of *S. minnesota* (*see* Fig. 1) was used (kindly provided by H. Brade, Biochemische Mikrobiologie, Forschungsinstitut Borstel, FRG). It was extracted from bacteria cultivated at 37°C by the phenol/chloroform/petrol ether method (Galanos, Lüderitz & Westphal, 1969), electrodialyzed (Galanos & Lüderitz, 1975) and lyophilized. For the simulation of the natural PL composition of the inner leaflet of the outer membrane, as determined for *Salmonella typhimurium* (Osborn et al., 1972), phosphatidylethanolamine (PE) from *E. coli*, phosphatidylglycerol (PG) from egg yolk, and cardiolipin (CL) from bovine heart were mixed in a molar ratio of 81 : 17 : 1.6. These phospholipids as well as phosphatidylcholine-dioleoyl (DOPC), phosphatidylcholine-β-arachidonoyl-γ-stearoyl and phosphatidylcholine type III S from soybean were purchased from Sigma (Deisenhofen, FRG) and used without further purification. The fatty acid pattern of soybean PC and the PL mixture was determined by GC-MS.

Phospholipids were dissolved at a concentration of 2 mg/ml in chloroform. LPS was dissolved either in PCP (90 g phenol in 10 g water, 15% chloroform and 5% petrol ether by vol) or in chloroform. A clear solution of LPS in chloroform was obtained by heating the LPS/chloroform mixture to 80°C for a few minutes. The α-toxin from *S. aureus* (mol wt = 34,000; activity 19 binding U/mg (29 binding U/mg protein)) was purchased from Calbiochem (Frankfurt, FRG) and dissolved at a concentration of 5 mg/100 μl in the respective membrane bathing solutions, which were unbuffered and contained (if not otherwise indicated) 100 mM NaCl and 10 mM $MgCl_2$. These stock solutions were stored in a deep freezer.

Preparation of Membranes

Planar membranes were formed from monolayers that were prepared in Teflon compartments with a surface area of 1 cm^2 and a height of 1 cm each, separated by a thin Teflon foil (d = 12.5 μm; Angst and Pfister, Zürich, CH) in which a small aperature (radius r between 50 and 150 μm) was punched. Prior to use the Teflon partitions were pretreated with hexadecane.

The aqueous solutions in both compartments could be stirred by means of magnetic bars and the test cell was thermostated electrically.

Monolayers were obtained either by spreading up to 5 μl of the lipid solutions on the aqueous phases in the compartments (Montal & Mueller, 1972) or from vesicle solutions according to the Schindler technique (Schindler, 1980).

Vesicle solutions were always freshly prepared by suspending a dried film of the lipids with the bathing solution and sonification for about 1 min (Branson sonifier) at room temperature. Lipid concentrations in the vesicle solutions were 1 mg/ml for PL and 5 mg/ml for LPS.

The time between spreading the monolayers or filling the compartments with the vesicle solutions, respectively, and the membrane formation by raising successively the levels of the bathing solutions above the aperture was at least 5 min. To avoid a bulging of the membranes, the subphase on both sides of the membranes were raised always to the same level.

Formation of the monolayers from the vesicle solutions and their equilibration was tested separately by surface tension measurements with a Wilhelmy ring balance (Krüss, Hamburg, FRG).

Electrical Measurements

For the electrical measurements the two compartments were connected via a single pair of Ag/AgCl electrodes to a function generator (Enertec Schlumberger, St Etienne, France) and to a current-voltage (I/V) converter, respectively, which consisted of an OPA 111 (Burr Brown, Filderstadt, FRG) operational amplifier and a 5 $\times$ 10^8 Ω feedback resistor.

Conductance measurements were carried out by clamping the transmembrane voltage with the described setup to a given value. The output voltage of the I/V converter (i.e., the transmembrane current) was displayed on an oscilloscope and recorded, if necessary after further amplification, on a strip chart recorder (Linseis, Selb, FRG).

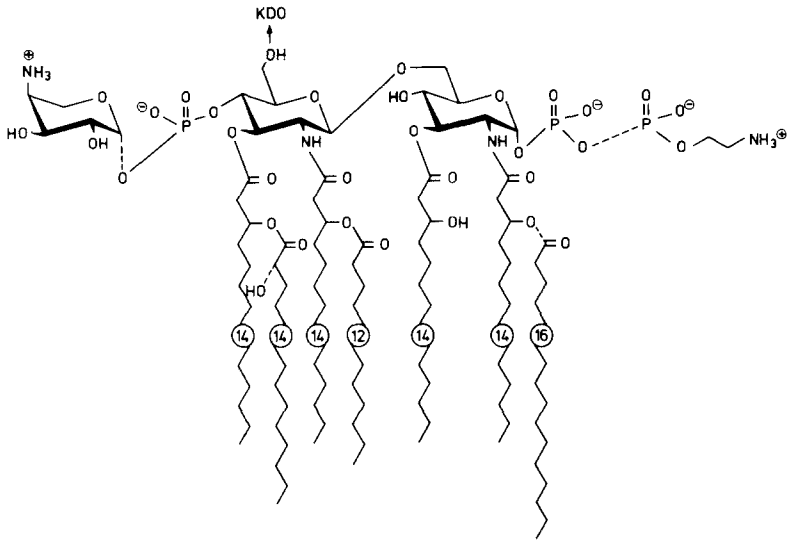

Fig. 2. Chemical structure of lipid A from *Salmonella minnesota*. Encircled numbers indicate the number of carbon atoms in the acyl chains. Dotted lines indicate a nonstoichiometric substitution

Voltage polarities refer to the potential of the electrode, which was virtually grounded by the operational amplifier, and the corresponding compartment of the test cell will be called the *cis* compartment.

For the determination of membrane capacitance a modification of the voltage-induced capacitance relaxation method (Benz & Janko, 1976) was used, which allows a continuous and sensitive (about 10^{-3} of the absolute capacitance) recording of the capacitance. Reliability of the method was checked with a number of membrane-analogous test circuits.

Values for the capacitance were obtained by subtracting the intrinsic capacitance of the Teflon partition, varying between 15 and 20 pF, from the measured total capacitance. Specific membrane capacitances C_M were calculated as the quotient of the membrane capacitance and the aperture area. The apertures were always circular and their area was determined microscopically with a typical error of less than 5%.

Results

General Membrane Characterization by Capacitance Measurements

Planar phospholipid bilayer systems have been described in their physical behavior in detail (for review *see*, e.g., White, 1986). In this report, lipopolysaccharides are major constituents of the bilayers. These amphiphiles differ considerably in many of their physico-chemical properties from that of phospholipids. Therefore, it was necessary to characterize the LPS-containing systems—asymmetric PL/LPS and symmetric LPS/LPS systems—in detail with respect to thickness, stability, properties of the boundary between the planar bilayers and the Teflon partition and a possible influence of solvent.

Thus, we performed a series of experiments in which the preparation method of the monolayers, the aperture radii and the temperature and composition of the membrane systems were varied. The influence of the various parameters has been monitored via capacitance measurements.

In Fig. 3 the specific capacitances of various reconstituted planar membrane systems are plotted *vs.* the average number N of carbon atoms of opposing hydrocarbon chains of the bilayer acyl moiety. The parameter N is defined for the symmetric membranes as twice the sum of the mean chain length, calculated with the relative abundances of each fatty acid residue in the total hydrocarbon mixture as weighting factors, and is taken as a measure for the membrane thickness. For the asymmetric membrane systems, N is calculated as the sum of the mean chain lengths, which are determined for each membrane leaflet separately. In this calculation, however, other properties of the acyl chains, especially the number of double bonds, which was shown to influence the bilayer thickness (Benz & Janko, 1976) are neglected. Nevertheless, as should be expected, the specific capacitances of membranes reconstituted by the same preparation method decrease with increasing N in a similar way as with increasing chain length (Benz et al., 1975).

A membrane thickness scale is also included in Fig. 3. Membrane thicknesses d_m were calculated under the assumption of the parallel plate condenser model ($C_M = \varepsilon_O \cdot \varepsilon_m/d_m$) with $\varepsilon_m = 2.1$ and $\varepsilon_O = 8.85 \times 10^{-12}$ F · m^{-1} (Läuger, 1966).

Specific capacitances of symmetric membranes from phospholipids obtained by the vesicle method (systems S1–S4) were roughly 15% higher than the

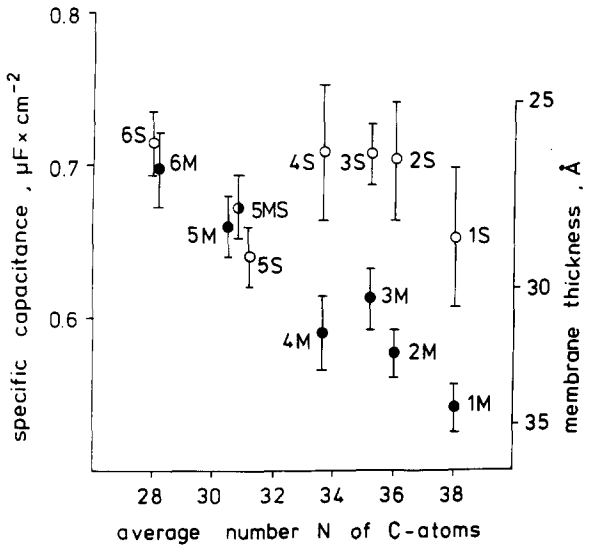

Fig. 3. Plot of specific capacitance and thickness of various reconstituted membrane systems *vs.* the average number N of carbon atoms of the opposing bilayer acyl chains. The different symbols indicate the technique used for the monolayer preparation: ○ from vesicle derived monolayers (*S*); ● from spread monolayers (*M*); and ◑ from a combination of both techniques (*MS*). Data are mean values of at least 10 independent measurements and error bars represent standard deviations of capacitance values. Membrane bathing solutions contained 100 mM NaCl and were unbuffered. Apertures had radius of 100 μm. The membrane systems under investigation were built up from: 1: PC-β-arachidonoyl-γ-stearoyl; 2: PC-dioleoyl; 3: PC from soybean type III S; 4: PL mixture (*see* Materials and Methods); 5: asymmetric membranes from R595 LPS and PL mixture (LPS monolayers were vesicle derived); 6: membranes built up from R595-LPS as the only component. The systems 5 and 6 were prepared at a temperature of 37°C, the others at room temperature (23°C)

values obtained for these membranes formed from spread monolayers.

Some additional observations made in connection with the capacitance measurements should be pointed out. In agreement with other authors (Niles, Levis & Cohen, 1988), we found that directly after the formation (successive raising of the monolayers above the aperture) of a membrane its capacitance varied for a considerable time period, and a stationary value, which then remained constant over some hours, is reached only after some minutes. These observations were made for all preparation methods and lipids used in our investigations. The results shown in Fig. 3 refer in all cases to stationary values.

In a series of experiments in which the aperture radius was varied (without changing the lipid and the preparation method), we found that the specific capacitance, here calculated as quotient of measured capacitance and aperture area, shows a slight but significant decrease with decreasing radius.

This is demonstrated in Fig. 4 for membranes spread from phosphatidylcholine-β-arachidonoyl-γ-stearoyl. The solid curve in this figure was obtained from a theoretical expression fitted to the experimental data. For this, it was assumed that an ideal bilayer in the center of the aperture is surrounded by a transition zone (annulus or torus) to the by three orders of magnitude thicker partition which, according to its large thickness, does not contribute to the total membrane capacitance. Therefore, using the aperture area for calculation of the specific capacitances the bilayer area is overestimated. As an approximation, the following dependence of the specific membrane capacitance on the aperture radius is found:

$$C_M(r) = C_M^{\infty} \cdot \left(1 - \frac{2 \cdot \delta r}{r} + \frac{\delta r^2}{r^2}\right) \text{ for } r \geq \delta r. \qquad (1)$$

r is the aperture radius and δr the width of the transition zone, taken as the thickness of the Teflon partition. C_M^{∞} is the true specific capacitance of the bilayer, or the capacitance measured with an aperture of infinite radius for which the contribution of the annulus is negligible. For the fit δr was taken as the thickness of the Teflon partition (12.5 μm) and C_M^{∞} was calculated from Eq. (1).

When membranes, which were prepared above their β-α phase transition (between the gel and the liquid-crystalline states of the hydrocarbon chains), were heated up to higher temperatures ($\Delta T \approx 20$°C), a decrease in membrane capacitance accompanied by fluctuations was observed. The capacitance reached a new stationary value and returned, in most experiments, to the original value (referred to the starting temperature) when the compartments were cooled down again.

For the LPS-containing membranes, which were prepared at 37°C, we observed, in general, a breakdown of the membranes when the temperature of the test chamber was reduced to 30°C. This temperature is slightly lower than that of the β-α chain melting transition of this deep rough mutant LPS (Brandenburg & Seydel, 1984). All attempts to rebuild a stable membrane below this temperature failed.

At 37°C, the asymmetric membranes were stable for up to some hours. This is comparable to the lifetimes generally achieved for symmetric phospholipid membranes. Also, electrical breakdown voltages (200–400 mV) and specific conductances ($10^{-8} - 2 \times 10^{-7}$ S · cm^{-2}) were not significantly different to those of pure phospholipid membranes.

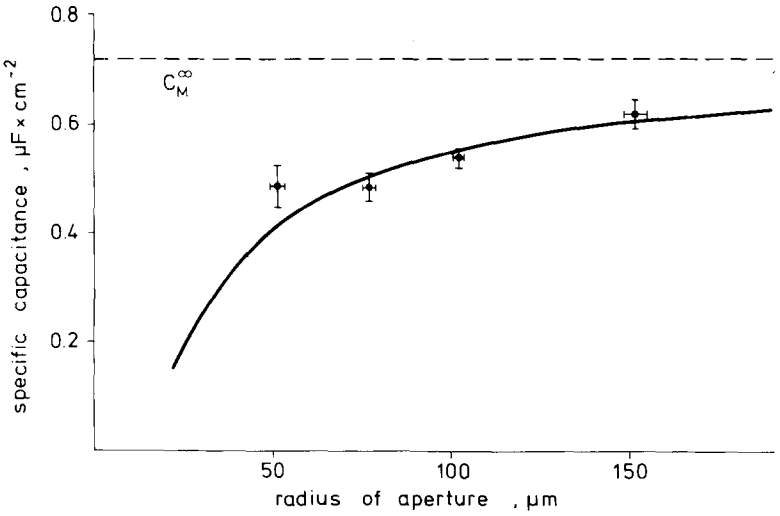

Fig. 4. Plot of specific capacitance, calculated as quotient of membrane capacitance and aperture area, for membranes spread from PC-β-arachidonoyl-γ-stearoyl *vs.* aperture radius. The continuous line was obtained from a theoretical expression (*see* Eq. (1)) fitted to the experimental data, which were obtained in each case from 30 measurements of stationary capacitance values

The use of $MgCl_2$ in the bathing solution in the millimolar range tended to improve as well the stability of the membranes as the formation process itself, in accordance with observations of a stabilizing effect of divalent cations—like Mg^{2+}—on the organization of outer membranes in bacterial cells (Nikaido & Vaara, 1985).

It should be mentioned that PL/LPS bilayers could be successfully formed also with LPS having a more complete inner core region (up to rough mutant LPS Rd_1, *see* Fig. 1).

Action of *S. aureus* α-Toxin

To demonstrate some distinct characteristics of the asymmetric membranes (one leaflet made from LPS, the other from the PL mixture) as compared to symmetric phospholipid membranes, we investigated the action on these membranes of α-toxin from *S. aureus,* which is known to form pores in the latter systems.

These experiments were performed exclusively with membranes prepared according to the Montal-Mueller technique because this way an uncontrollable interaction of the protein with vesicles in the subphase, which are a prerequisite of the Schindler technique, could be avoided.

In first experiments we found even at the relatively high concentrations applied that the α-toxin was not active at all on symmetric LPS membranes. However, in accordance with measurements reported by Menestrina (1986), it was active on symmetric membranes, which were made from the PL mixture.

These basic observations are shown in Fig. 5 (traces *A* and *B*) for the experimental conditions: aperture radius 50 μm and a toxin concentration of 250 μg/ml bathing solution. The respective experimental situation is illustrated by the inserts at each trace in this and the following figure. After the addition of α-toxin the compartments were stirred rigorously for a short time (≈20 sec). For membranes spread from the PL mixture (Fig. 5, trace *A*) less than 1 min after the addition of α-toxin, a stepwise increase of the transmembrane current due to the pore formation was observed.

The amplitudes of the single current steps were of rather homogenous size (roughly 2 pA in 100 mM NaCl and at a transmembrane potential clamped to 20 mV corresponding to a flow rate of about 1.2×10^7 ions · sec^{-1}). Under the assumption that the pore is of cylindrical shape (length l and radius r) and filled with the membrane bathing solution, the conductance G of a single pore is related to the geometry of the pore and the conductivity σ of the bathing solution by:

$$G = \frac{I_P}{U_M} = \frac{\pi \cdot r^2 \cdot \sigma}{l} \tag{2}$$

where I_P is the current through a single pore at the transmembrane potential U_M. The described experiment yielded a pore radius of ≈0.85 nm, if l was taken as the thickness of the membranes (≈3 nm) as derived from the capacitance measurements (the influence of the transition zone was not taken into account for the calculation of the membrane thickness).

In accordance with observations by Menestrina et al. (1987) on the pore formation by *E. coli* α-toxin in planar bilayers from a mixture of PC and PE as well as from POPC (1,2-palmitoyloleoyl-phosphati-

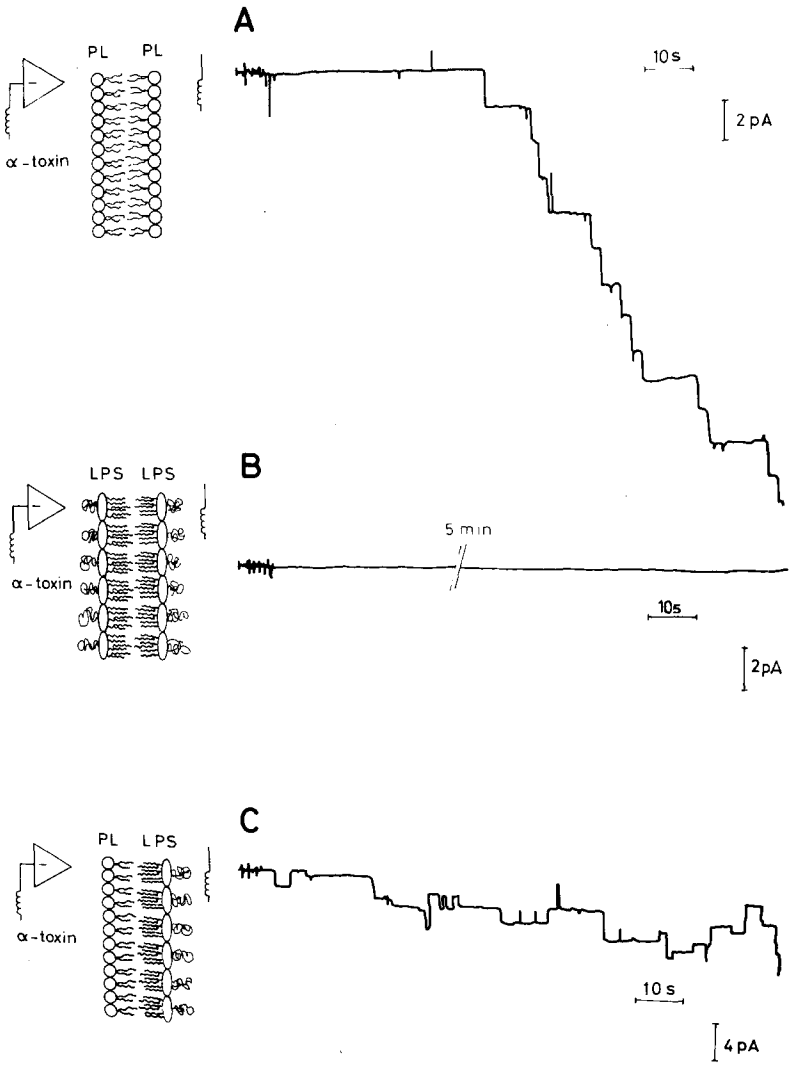

Fig. 5. Microscopic conductance changes of voltage-clamped planar membranes of different lipid composition after the addition of α-toxin. All membranes were spread from chloroform solutions on a 100 mM NaCl subphase. To achieve low noise of the current records, apertures with 50-μm radius were used. Transmembrane potential was clamped to −20 mV. After the addition of α-toxin to the *cis* side (i.e., virtual ground) in amounts of 5 μl from the stock solutions (according to toxin concentration of 250 μg/ml bathing solution) the compartments were stirred for a short time. (*A*) Membranes built up from the PL mixture. (*B*) Membranes built up from R595-LPS as the only component. The double bar indicates a period of 5 min in which the transmembrane current did not change. (*C*) Asymmetric membranes with the toxin added to the PL side. The respective experimental situation is illustrated by the inserts at each trace in this and in Fig. 6.

dylcholine), also in our experiments with *S. aureus* α-toxin, membranes broke down when a large number of pores was incorporated into the bilayer.

Under the same experimental conditions, concerning transmembrane potential, bathing solution and amount of toxin added, no effect was observed for symmetric LPS membranes (Fig. 5, trace *B*).

For the asymmetric PL/LPS membranes two different behaviors were observed, depending on the compartment to which the α-toxin was added. When the toxin was added to the PL side of the membrane (*cis* compartment), again changes in the transmembrane current were observed (Fig. 5, trace *C*). However, these changes differ from those for the symmetric PL membranes, in that the current decreased statistically for some steps or changed between two conductance levels. Such terminating steps in the current traces, which presumably result from a closure of single pores, were very rare events when the toxin acted on PL membranes. When the toxin was added to the LPS side, no change of the transmembrane current was observed (*result not shown*).

To measure the rate of pore formation for the different membrane systems, we have used larger aperture radii (75–100 μm) and recorded the current traces at lower sensitivity. The results of these macroscopic current measurements are shown in Fig. 6 demonstrating that the rate of pore formation, as monitored by the conductance measurements, is significantly lower in the asymmetric LPS/PL than in the symmetric PL membranes. The dependence of the rate of pore formation in both systems on the toxin concentration gives evidence for a power dependence larger than one, as would be expected from the fact that the conducting pores are formed

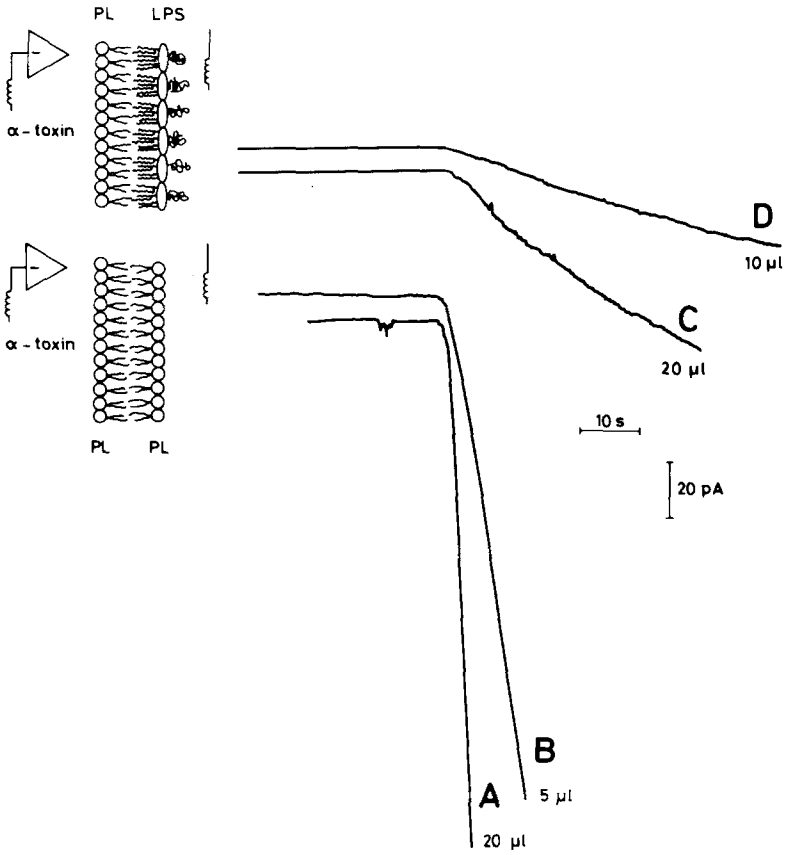

Fig. 6. Records of macroscopic current for symmetric membranes spread from PL mixture (traces *A* and *B*) as well as for asymmetric membranes from R595-LPS and PL mixture (traces *C* and *D*) after the addition of various amounts of the α-toxin stock solutions (5 µl correspond to 250 µg α-toxin) to the *cis* side (in the case of asymmetric membranes, the PL side was *cis*). Membrane bathing solutions contained 10 mM $MgCl_2$. All experiments were performed with the same Teflon partition with an aperture radius of 75 µm

by hexamers via the aggregation of α-toxin monomers (*compare*, e.g., Menestrina, 1986; Bhakdi & Tranum-Jensen, 1988).

Discussion

In this paper we have shown that the established method of Montal and Mueller (1972) for the formation of planar membranes from monolayers and its modification by Schindler (1980) can be successfully applied to the preparation of asymmetric membranes from LPS on one side and PL on the other side. These asymmetric planar membranes represent a new reconstitution system as it contains a particular class of amphiphiles, the lipopolysaccharides, which differ considerably in many of their physico-chemical properties from those of phospholipids.

A characterization of these reconstituted asymmetric membranes is principally feasible via the electrical parameters (capacitance, conductance and breakdown voltage) in comparison to the respective data from pure PL systems.

In Fig. 3 the data for the specific capacitance of various membrane systems are presented showing that for symmetric PL membranes (1–4) there are significant differences in the specific membrane capacitances depending on the preparation method, whereas these differences were not observed for the symmetric LPS and the asymmetric PL/LPS systems. As known from literature (e.g., Benz et al., 1975), the solvent tends to reduce specific membrane capacitances and presumably the observed lower capacitances of membranes from chloroform-spread monolayers are caused by chloroform remaining in the monolayers. The fact that this dependence on the preparation method is not observed for the symmetric LPS and the asymmetric PL/LPS systems may be explained by the comparatively high packing density of the hydrocarbon moiety of LPS. From monolayer measurements the limiting area of a LPS molecule of *S. minnesota* R595 was determined to 1.05 nm^2 (Brandenburg & Seydel, 1984) and for DOPC this value is 1.1 nm^2 (*our unpublished data*), each at a surface pressure of 25 $mN \cdot m^{-1}$. This corresponds to a factor of approximately 0.95, whereas the ratio of the number of fatty acid residues is 3 to 3.5 depending on the number of substituents (*compare* Fig. 2).

The capacitance value of $C_M = (0.71 \pm 0.02)\ \mu F \cdot cm^{-2}$ taken from Fig. 3 for vesicle-derived membranes from soybeam PL (system 3S) is in good agreement with $C_M = (0.75 \pm 0.05)\ \mu F \cdot cm^{-2}$ reported by Schindler (1980), and the value of $C_M = (0.58 \pm 0.02)\ \mu F \cdot cm^{-2}$ for membranes spread from DOPC with the respective value of $C_M = (0.624 \pm 0.011)\ \mu F \cdot cm^{-2}$ given by Benz and Janko (1976) for DOPC BLM's with *n*-hexadecane as solvent. The specific capacitance values for the LPS-containing membranes are reasonable, when compared with the respective value of pure phospholipid systems by considering the differences in the hydrocarbon chain lengths.

The other two electrical parameters, breakdown voltage and conductance, show for all systems relative large variations between different preparations. These parameters seem, therefore, to be unsuitable for a reliable characterization of different membrane systems according to the preparation technique and lipid composition.

From the agreement in the electrical capacitance values for the asymmetric membrane systems prepared by the two different techniques we decided to utilize, in further experiments, exclusively the Montal-Mueller technique, because this technique requires considerably lower amounts of lipids.

The observed dependence of the specific capacitance on the radius of the aperture as illustrated in Fig. 4 is in reasonable agreement with the calculated dependence based on the assumption of a toroidal transition zone (solid line in Fig. 4). The existence of such a transition zone for planar bilayers prepared from monolayers, which is presumably built up from the hydrocarbon used for the pretreatment of the Teflon partition, has recently been observed directly by Niles et al. (1988).

The decrease of membrane capacitance with increasing temperature might reflect a similar behavior as that reported by White (1986) for BLM's. According to the measurements of White, the solubility of alkanes like hexadecane in the bilayer is strongly temperature dependent in a way that it increases with increasing temperature. Thus, the membrane capacitance decreases during the heating scan, since a larger amount of solvent penetrates the bilayer from the transition zone and the membrane becomes thicker.

The observed dependences of the specific capacitances as well on the aperture radius as on temperature give evidence that bilayers made from monolayers are not at all solvent free.

The symmetric LPS and the asymmetric PL/LPS systems contained on two or on one side, respectively, exclusively lipids with fully saturated fatty acid residues. These membrane systems could be formed, and were stable, only at temperatures above the β-α chain melting transition temperature T_C (gel to liquid-crystalline transition). This might be explained by a low overall membrane flexibility caused by the high degree of order of the hydrocarbon moiety in the gel state.

The electrical characterization of the asymmetric LPS/PL planar membrane systems demonstrates that these systems behave, except for some distinct properties, analogously to PL systems comprehensively described in literature (for review *see*, e.g., White, 1986). These distinct properties are, e.g., breakdown at temperatures below T_C and no solvent influence on membrane capacitance. The latter observation can possibly be explained by the primary chemical structure and the physical conformation of the LPS molecule.

To demonstrate that the particular lipid composition of the LPS-containing asymmetric membranes shows a particular behavior in the interaction with membrane-active molecules, we compared the action of the pore-forming α-toxin of *S. aureus* on symmetric PL with that on symmetric LPS and on asymmetric LPS/PL planar membrane systems.

The mechanism of action of α-toxin on mammalian cells and phospholipid model membrane systems have been described in literature (Menestrina, 1986; Bhakdi & Tranum-Jensen, 1988). With our present studies we could show that the α-toxin of *S. aureus*, contrary to the observations with phospholipid membranes, does not lead to a pore formation (Fig. 5, trace *B*) with LPS/LPS membranes and that the rate of pore formation of this toxin in an asymmetric PL/LPS bilayer, where the toxin is added to the PL side, is reduced as compared to a symmetric PL bilayer. These observations were made at relatively high toxin concentrations, which were applied to demonstrate qualitatively differences in its activity on the different membrane systems. Nevertheless it is unlikely that these observations are attributed to artifacts (e.g., contaminations of the protein) because the amplitudes of the single-channel conductances measured after the addition of the toxin to the PL/PL systems are in agreement with respective data from Menestrina (1986).

The fact that no conductance increase was observed after the addition of α-toxin to symmetric LPS membranes (Fig. 5*B*) and that a significantly slower increase, including terminating steps, after the addition of the toxin to the PL side of an asymmetric PL/LPS bilayer occurred (Fig. 5*C* and Fig. 6), allows only the conclusion that in these cases no or, respectively, less conducting pores are formed, but not that no toxin was incorporated. Presently

we cannot decide whether these observations are due to different surface charges or to the comparatively high state of order of deep rough mutant LPS membrane systems at physiological temperature—referred to membranes made from a PL mixture resembling the natural composition of the inner leaflet of the outer membrane of Gram-negative bacteria (Brandenburg & Seydel, 1988). Also the sugar moiety could possibly influence sterically (inhibit) the pore formation.

The modulation of the activity of a bacterial toxin on planar membranes by their lipid composition has previously been demonstrated also by Donovan, Simon and Montal (1982) who studied the insertion of diphtheria toxin into phosphoinositide-containing asymmetric membranes. They found that for optimal activity of the toxin inositide was required on the side of the membrane opposite to that in which diphtheria toxin was introduced.

The reconstitution model of the outer membrane of Gram-negative bacteria presented in this report is a powerful tool for studying, first of all, properties of the unmodified lipid matrix with respect to its barrier function and drug susceptibilities, and the action of newly developed drugs. It will, furthermore, allow the investigation of functional properties of outer membrane proteins in the native lipid environment.

We are indebted to G. von Busse for his skillful technical assistance, to Dr. H. Brade for supplying the lipopolysaccharide, and to Prof. Dr. E.T. Rietschel for many fruitful discussions. We also thank Mss. M. Lohs and B. Köhler for preparing the drawings and photographs, respectively.

References

Benz, R., Fröhlich, O., Läuger, P., Montal, M. 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta* **394:**323–334

Benz, R., Janko, K. 1976. Voltage-induced capacitance relaxation of lipid bilayer membranes. Effects of membrane composition. *Biochim. Biophys. Acta* **435:**721–738

Bhakdi, S., Tranum-Jensen, J. 1988. Damage to cell membranes by pore forming bacterial cytolysins. *In:* Progress in Allergy. K. Ishizaka, P. Kallos, P.J. Lachmann, and B.H. Waksman, editors. pp. 1–43. Karger, Basel

Brandenburg, K., Seydel, U. 1984. Physical aspects of structure and function of membranes made from lipopolysaccharides and free lipid A. *Biochim. Biophys. Acta* **775:**225–238

Brandenburg, K., Seydel, U. 1988. Orientation measurements on membrane systems made from lipopolysaccharides and free lipid A by FT-IR spectroscopy. *Eur. Biophys. J.* **16:**83–94

Donovan, J.J., Simon, M.I., Montal, M. 1982. Insertion of diphtheria toxin into and across membranes: Role of phosphoinositide asymmetry. *Nature (London)* **298:**669–672

El-Falaha, B.M.A., Russell, A.D., Furr, J.R. 1983. Sensitivities of wild-type and envelope-defective strains of *Escherichia coli* and *Pseudomonas aeruginosa* to antibacterial agents. *Microbios* **38:**99–105

Galanos, C., Lüderitz, O. 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. *Eur. J. Biochem.* **54:**603–610

Galanos, C., Lüderitz, O., Westphal, O. 1969. A new method for the extraction of R Lipopolysaccharides. *Eur. J. Biochem.* **9:**245–249

Lüderitz, O., Freudenberg, M.A., Galanos, C., Lehmann, V., Rietschel, E.Th., Shaw, D.H. 1982. Lipopolysaccharides of Gram-negative bacteria. *Curr. Top. Membr. Transp.* **17:**79–149

Läuger, P., Lesslauer, E.M., Richter, J. 1966. Electrical properties of bimolecular phospholipid membranes. *Biochim. Biophys. Acta* **135:**20–32

Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.* **90:**177–190

Menestrina, G., Mackman, N., Holland, I.B., Bhakdi, S. 1987. *Escherichia coli* hemolysin forms voltage-dependent ion channels in lipid membranes. *Biochim. Biophys. Acta* **905:**109–117

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **12:**3561–3566

Nikaido, H. 1979. Die Permeabilität der äusseren Bakterienmembran. *Angew. Chem.* **91:**394–407

Nikaido, H., Vaara, M. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49:**1–32

Niles, W.D., Levis, R.A., Cohen, F.S. 1988. Planar bilayer membranes made from phospholipid monolayers form by a thinning process. *Biophys. J.* **53:**327–335

Osborn, M.J., Gander, J.E., Parisi, E., Carson, J. 1972. Mechanism and assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* **247:**3962–3972

Rietschel, E.T., Brade, H., Brade, L., Brandenburg, K., Schade, U., Seydel, U., Zähringer, U., Galanos, C., Lüderitz, O., Westphal, O., Labischinski, H., Kusumoto, S., Shiba, T. 1987. Lipid A, the endotoxic center of bacterial lipopolysaccharides: Relation of chemical structure to biological activity. *In:* Proceedings of 2nd International Conference of Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test. A. Watson, editor. pp. 25–53. A.R. Liss, New York

Rietschel, E.T., Wollenweber, H.W., Brade, H., Zähringer, U., Lindner, B., Seydel, U., Bradaczek, H., Barnickel, G., Labischinki, H., Giesbrecht, P. 1984. Structure and conformation of the lipid A component of lipopolysaccharide. *In:* Handbook of Endotoxin. Vol. 1, pp. 187–220. Chemistry of Endotoxin. E.T. Rietschel, editor. Elsevier, Amsterdam

Schindler, H. 1980. Formation of planar bilayers from artificial or native membrane vesicles. *FEBS Lett.* **122:**77–79

White, S.H. 1986. The physical nature of planar bilayer membranes. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 3–32. Plenum, New York—London

Received 23 September 1988; revised 3 January 1989