J. Membrane Biol. 162, 127–138 (1998)

The Journal of
Membrane
Biology

Molecular Mechanisms of Polymyxin B-Membrane Interactions: Direct Correlation Between Surface Charge Density and Self-Promoted Transport

A. Wiese[1], M. Münstermann[1], T. Gutsmann[1], B. Lindner[1], K. Kawahara[2], U. Zähringer[1], U. Seydel[1]

[1]Research Center Borstel, Center for Medicine and Biosciences, Department of Immunochemistry and Biochemical Microbiology, Parkallee 10, D-23845 Borstel, Germany

[2]The Kitasato Institute, Department of Bacteriology, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan

Received: 16 September 1997/Revised: 25 November 1997

Abstract. We have studied the interaction of the polycationic peptide antibiotic polymyxin B (PMB) with asymmetric planar bilayer membranes via electrical measurements. The bilayers were of different compositions, including those of the lipid matrices of the outer membranes of various species of Gram-negative bacteria. One leaflet, representing the bacterial inner leaflet, consisted of a phospholipid mixture (PL; phosphatidylethanolamine, -glycerol, and diphosphatidylglycerol in a molar ratio of 81:17:2). The other (outer) leaflet consisted either of lipopolysaccharide (LPS) from deep rough mutants of PMB-sensitive (*Escherichia coli* F515) or -resistant strains (*Proteus mirabilis* R45), glycosphingolipid (GSL-1) from *Sphingomonas paucimobilis* IAM 12576, or phospholipids (phosphatidylglycerol, diphytanoylphosphatidylcholine). In all membrane systems, the addition of PMB to the outer leaflet led to the induction of current fluctuations due to transient membrane lesions. The minimal PMB concentration required for the induction of the lesions and their size correlated with the charge of the lipid molecules. In the membrane system resembling the lipid matrix of a PMB-sensitive strain (F515 LPS/PL), the diameters of the lesions were large enough ($d = 2.4$ nm ± 8%) to allow PMB molecules to permeate (self-promoted transport), but in all other systems they were too small. A comparison of these phenomena with membrane effects induced by detergents (dodecyltriphenylphosphonium bromide, dodecyltrimethylammonium bromide, sodiumdodecylsulfate) revealed a detergent-like mechanism of the PMB-membrane interaction.

Key words: Polymyxin B — Planar lipid bilayer — Outer membrane — Membrane lesions — Surface charge — Resistance

Correspondence to: U. Seydel

Introduction

Polycationic peptides/proteins, either synthetic or natural isolates, are of increasing interest as potent antibacterial agents, (e.g., Hirata et al., 1995; Bucklin et al., 1995; Levy et al., 1995; Vaara & Porro, 1996; Ried et al., 1996; Gough, Hancock & Kelly, 1996). Polymyxin B (PMB) is one of the most investigated polycationic peptides, but exerts beside its antibacterial activity also severe toxic side effects (Thelestam & Mollby, 1980). For the development and application of new peptide structures devoid of the undesirable side effects, the knowledge of the general molecular mechanisms underlying the interaction between the drugs and the target membrane is an essential prerequisite. To this end we have studied the induction of membrane lesions by PMB in differently composed planar lipid bilayers.

Polymyxins are antibiotics isolated from various strains of *Bacillus polymyxa*. They are bactericidal to Gram-negative bacteria and the mode of action comprises two major steps, binding to and permeabilization of the outer membrane and induction of lethal leakages in the cytoplasmic membrane for cytoplasmic components (Vaara, 1992). The outer membrane represents a permeation barrier for hydrophobic antibiotics. This is due to the fact that, other than the inner leaflet which is composed of a mixture of phospholipids, the outer leaflet is built up exclusively of a glycolipid, normally lipopolysaccharide (LPS) (Nikaido & Vaara, 1985). In 1991, it was found by Kawahara et al. (1991) that in the outer leaflet of the outer membrane of the Gram-negative species *Sphingomonas paucimobilis* IAM 12576 (formerly

Pseudomonas paucimobilis) LPS is completely substituted by glycosphingolipids (GSL). Both glycoconjugates, LPS and GSL, are highly variable in their glycostructures but the chemical structures of their respective lipid anchors are relatively conserved. LPS consists of an oligo- or polysaccharide portion which is covalently linked to a lipid component, termed lipid A, which anchors the molecule in the membrane (Rietschel et al., 1994). Depending on the species and/or strain from which the LPS was obtained, each LPS molecule carries different numbers of negative charges as carboxyl or phosphoryl groups. In GSL, the hydrophobic moiety is heterogeneous with respect to the dihydrosphingosine residue but is, in each case, quantitatively substituted by an amide-bound (S)-2-hydroxymyristic acid at the dihydrosphingosine. The sugar moieties of the two main fractions GSL-4A and GSL-1 consist of a Man-Gal-GlcN-GlcA tetrasaccharide and a GlcA monosaccharide, respectively (Kawahara et al., 1991). In the present paper, only the glycolipids with the shortest sugar moieties have been used, i.e., deep rough mutant LPS (Re LPS) carrying two Kdo units at the lipid A and GSL-1.

Incorporated into these extremely asymmetric lipid matrices are water-filled channels, the so-called porins, which allow the diffusion of small hydrophilic compounds with a molecular weight up to about 600 Da (Nakae, 1976; Nikaido & Vaara, 1985; Benz & Bauer, 1988). The polymyxin B (PMB) used for the present investigations (a mixture of polymyxins B_1 and B_2) had an average molecular weight of about 1200 Da (determined mass spectrometrically) and 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 (Storm & Rosenthal, 1977; El Mashak & Tocanne, 1980). As PMB is thus inhibited from diffusing through the porin channels, it must permeate through the lipid matrix to reach its final target, the cytoplasmic membrane (Vaara, 1992).

Gram-negative bacteria, in general, are known to be most sensitive towards the antibacterial action of PMB. There are, however, also species and strains which are either *a priori* resistant or have been grown resistant to PMB, and this resistance could be shown to be associated with an extensive cationic substitution of LPS by 4-amino-4-deoxy-L-*arabino*-pentopyranose (L-Ara*p*4N) leading to a reduction of the net negative charge of LPS (Nummila et al., 1995; Helander et al., 1996; Seltmann, Lindner & Holst, 1996). This would imply that the negative molecular charge plays a decisive role in the sensitivity or resistance of a Gram-negative species or strain towards polycationic antibiotics. Data, however, have also been published in favor of a hydrophobic interaction of PMB with lipids (Srimal et al., 1996).

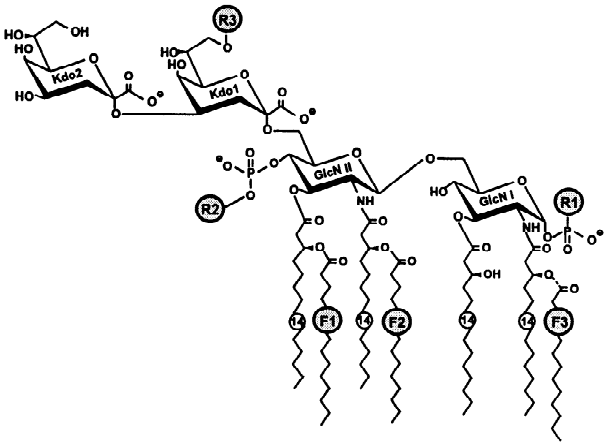
Planar lipid bilayers are a suitable tool for studying drug interactions with membranes. Early work on the membrane interaction of PMB goes back to Teuber and coworkers who investigated the interaction of PMB with black lipid membranes (BLM) made from various phospholipids differing in their surface charge density. They found that PMB led to membrane destabilization but not to penetration (Miller, Bach & Teuber, 1978).

The asymmetric outer membrane of Gram-negative bacteria is the primary target of PMB and other antibiotic peptides/proteins. BLM, besides containing solvent remainders, do not allow the preparation of asymmetric membranes and with that the reconstitution of the lipid matrix of the outer membrane. Therefore, we have adopted the method introduced by Montal and Mueller (1972) for the formation of solvent free symmetric and asymmetric planar bilayer membranes to electrically characterize membrane effects induced by membrane-active agents. The lipid composition of the planar bilayers can be chosen nearly at will allowing the reconstitution of mostly any symmetric or asymmetric bilayer provided that the lipids are soluble in chloroform and their hydrocarbon chains are in the liquid crystalline phase at 37°C, the temperature at which the experiments are performed. With these model membranes we have studied the influence of the lipid matrix, in particular that of the net negative charges of the composing lipids, but also that of their molecular size and with that of the surface charge density on the interaction of PMB. Because during this interaction the outer membrane undergoes a rapid increase in permeability for charged or polar molecules of low molecular weight (Vaara, 1992), an increase in ion conductivity, which is negligible for an undisturbed lipid bilayer, should be expected. Thus, the measurement of conductivity changes as a consequence of PMB-membrane interaction should provide information of the underlying mechanisms.

From earlier studies on the interaction of PMB and its nonapeptide (PMBN) lacking the fatty acid tail of PMB with different model membranes as well as lipid monolayers we proposed a detergent-like mechanism for the interaction of PMB with lipid bilayer membranes (Schröder, Brandenburg & Seydel, 1992). According to this model, PMB alone or together with lipid molecules of the matrix forms transient water-filled membrane lesions above a certain threshold concentration. In the case of asymmetric membranes made from the LPS of the deep rough mutant of *Salmonella enterica sv.* Minnesota strain R595 (R595 LPS), these lesions are large enough to allow the drug molecules to permeate the membrane ('self-promoted transport', Hancock, 1984). The particular detergent-like conformation of PMB molecules favors the formation of micellar structures in the lamellar lipid bilayer, which manifest as transient lesions. Membrane activity of PMB starts well below its critical aggregate concentration (CAC) as determined in

a membrane-free aqueous environment. This might be explained by electrostatic interaction between negatively charged membrane surfaces and the polycationic drug molecules leading to an accumulation of the latter at the membrane surface.

In continuation of these studies, the present investigations are focused on the elucidation of the influence of the lipid matrix on the formation and size of transient membrane lesions and on the mechanisms of PMB resistance. Of particular interest is the question how the molecular net charge or the surface charge density of the membrane surface influences pore formation. To this end, we investigated 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. One leaflet, representing the bacterial inner leaflet, consisted of a phospholipid mixture referred to as PL. The other (outer) leaflet consisted either of lipopolysaccharide (LPS) from deep rough mutants of PMB-sensitive (*Escherichia coli* F515) or — resistant strains (*Proteus mirabilis* R45) or of glycosphingolipid (GSL-1) from *Sphingomonas paucimobilis* IAM 12576. Other bilayers consisted also of PL on one side but of a phospholipid, phosphatidylglycerol (PG) or diphytanoylphosphatidylcholine (DPhyPC) on the other. The neutral DPhyPC was used instead of the negatively charged lipids to look more closely into the proposed accumulation of PMB at negatively charged membrane surfaces, because in this case PMB-induced lesions should be expected only at PMB concentrations above or close to its CAC. Furthermore, we investigated the interaction of detergents like sodiumdodecylsulfate (SDS), dodecyltriphenylphosphonium bromide (DTPPB), and dodecyltrimethylammonium bromide (DTMAB) with F515 LPS/PL bilayer membranes to verify our proposed model of a detergent-like mechanism underlying the PMB action.



	F515 LPS	R595 LPS	R45 LPS
R1	H	H ≥ 90% P-Etn ≤ 10%	H
R2	H	L-Arap4N 65% H 35%	H 50% L-Arap4N 50%
R3	H	H	H 50% L-Arap4N 50%
F1	14:0	14:0	14:0
F2	12:0	12:0	14:0
F3	H	H 70% 16:0 30%	H

Fig. 1. Chemical structures of the deep rough mutant lipopolysaccharides used in this study.

Materials and Methods

Lipids and Other Chemicals

For the formation of asymmetric LPS/PL membranes, deep rough mutant LPS from *Escherichia coli* strain F515 (F515 LPS) and from *Proteus mirabilis* strain R45 (R45 LPS) (chemical structures according to (Zähringer et al., 1985; Rietschel et al., 1992; Vinogradov et al., 1994; Rietschel et al., 1994, *see* Fig. 1) were used. LPS were extracted by the phenol/chloroform/petroleum ether method (Galanos, Lüderitz & Westphal, 1969), purified, lyophilized, and transformed into the triethylamine salt form. The amounts of non-stoichiometric substitutions by fatty acids, L-Ara*p*4N, and phosphoethanolamine (P-Etn) were analyzed by MALDI-TOF mass spectrometry. Thus, in the R595 LPS the 16:0 fatty acid linked to the amide-linked 3-OH-14:0 in position 2 of the reducing glucosamine was present only to 30% and L-Ara*p*4N linked to the 4′-phosphate to 65%. In R45 LPS, approximately 50% of the phosphates linked to the 4′ phosphate of lipid A and 50% of the first Kdo were substituted by L-Ara*p*4N.

The glycosphingolipid with the shortest sugar moiety, GSL-1, was used for the formation of GSL-1/PL membranes. GSL-1 was extracted from *S. paucimobilis* IAM 12576 with chloroform/methanol (Kawahara et al., 1991) and purified by silica gel column chromatography. LPS was not present in these preparations as was confirmed chemically by the lack of 3-OH fatty acids. In Fig. 2, the chemical structure of GSL-1 is depicted, showing that the hydrophobic part is heterogeneous with respect to the occurrence of two major sphingosine derivatives (*erythro*-1,3-dihydroxy-2-amino-octadecane and *erythro*-1,3-dihydroxy-2-amino-*cis*-13,14-methylene-eicosane), which are present in a ratio of approximately 1:4 (Kawahara et al., 1991).

Phosphatidylethanolamine (PE) from bovine brain (type I), phosphatidylglycerol (PG) from egg yolk lecithin (sodium salt), and diphosphatidylglycerol (DPG) from bovine heart (sodium salt) were purchased from Sigma (Deisenhofen, Germany), Diphytanoylphosphatidylcholine (DPhyPC) from Avanti Polar Lipids (Alabaster, AL). All phospholipids were used without further purification.

Polymyxin B was purchased from Sigma (Deisenhofen, Germany). Sodium-dodecylsulfate (SDS) was from Serva (Heidelberg, Germany), dodecyltriphenylphosphonium bromide (DTPPB) from EGA-Chemie (Steinheim, Germany). Dodecyltrimethylammonium bromide (DTMAB) was from Sigma.

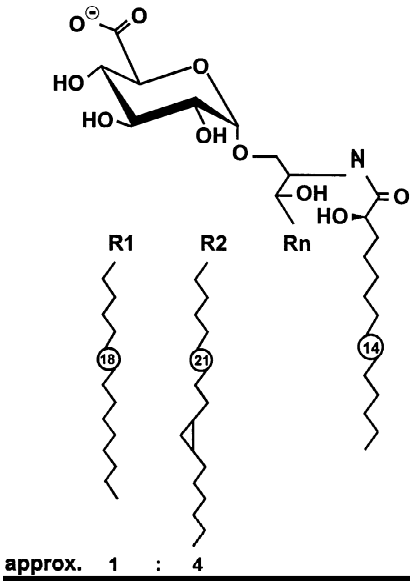


Fig. 2. Chemical structure of glycosphingolipid (GSL-1) from *S. paucimobilis* IAM 12576.

Determination of Molecular Charge and Surface Charge Density

The molecular charges were calculated assuming that phosphoryl and carboxyl groups represent one negative charge each and considering the nonstoichiometric substitutions by L-Ara*p*4N or P-Etn (*see* Fig. 1). In GSL-1, the carboxyl group of the glucuronic acid is known to carry on the average only 0.5 negative charges at physiological pH (Wiese et al., 1996).

The surface charge density is determined as the quotient of the average molecular charge and the effective molecular areas of the lipid molecules. The latter were determined from pressure-area isotherms obtained with a thermostated Langmuir film balance equipped with a Wilhelmy system. Monolayers were spread from 10^{-3} M chloroform/methanol (9:1 v/v) solutions of the lipids on an aqueous subphase containing 100 mM KCl, 5 mM $MgCl_2$ in HEPES buffer at pH 7. The experiments were run at 37°C. Prior to isotherm registration, monolayers were equilibrated at zero pressure for 5 min to allow evaporation of the solvent, compressed to 15 mN m^{-1} and allowed to equilibrate for 15 min. After that, the monolayers were expanded to zero pressure, allowed to equilibrate for 10 min, and the area-pressure isotherms were registered at a compression rate of 3 mm^2 sec^{-1}. The molecular areas of the lipids were then determined at a surface pressure of 30 mN m^{-1}, which is supposed to resemble the lateral pressure in a bilayer membrane under physiological conditions (Marcelja, 1974; Nagle, 1976; Blume, 1979; Ebara & Okahata, 1994).

Preparation of Planar Bilayers and Electrical Measurements

Planar bilayers according to the Montal-Mueller technique (Montal & Mueller, 1972) were prepared as described before (Seydel, Schröder, Brandenburg, 1989). Briefly, asymmetric bilayers were formed by apposing two lipid monolayers prepared on aqueous subphases (bathing solution) from chloroformic solutions of the lipids at a small aperture (typically 150 μm diameter) in a thin Teflon septum (12.5 μm thickness). The apparatus for membrane formation consisted of two Teflon compartments of 1.5 ml volume each. Prior to membrane formation, the septum was pretreated with a hexane/hexadecane mixture (20:1 v/v). For the formation of bilayer membranes, natural phospholipids and DPhyPC were dissolved in chloroform (2.5 mg/ml), GSL-1 (2.5 mg/ml) in chloroform/methanol (10:1 v/v) at room temperature, and LPS (2.5 mg/ml) in chloroform/methanol (10:1 v/v) and heated to 95°C for 2 min. The PL leaflet of asymmetric LPS/PL membranes consisted of a mixture of PE, PG, and DPG in a molar ratio of 81:17:2 resembling the phospholipid composition of the inner leaflet of the outer membrane of *Salmonella typhimurium* (Osborn et al., 1972).

For electrical measurements, planar membranes were voltage-clamped via a pair of Ag/AgCl-electrodes (type IVM E255, Advanced Laboratory Research, Franklin, MA) connected to the head stage of an L/M-PCA patch clamp amplifier (List-Medical, Darmstadt, Germany). In all cases, the compartment to which the peptide was added was grounded. Current was defined positive when cation flux was directed towards the grounded compartment. The clamp voltage was supplied from a built-in power supply. Membrane current and clamp voltage were stored using a digital-tape recorder (DTR 1202, Biologic, Claix, France). The stored signals were low pass filtered at 250 Hz with a 4-pole Bessel filter (Ithaco Model 4302, New York) sent to the microcomputer system and digitized with a PCI-20098C-1B analog input board (Intelligent Instrumentation, Leinfelden-Echterdingen, Germany). For the determination of the single fluctuation amplitudes, the current traces were filtered at a corner frequency of 2.5 kHz and digitized with a sampling frequency of 7 kHz.

All measurements were performed with a bathing solution consisting of 100 mM KCl and 5 mM $MgCl_2$ at a temperature of 37°C. To adjust to pH 7, the bathing solution was buffered with 5 mM HEPES, for pH 3 with 5 mM sodium citrate. In all cases the specific electrical conductivity of the bathing solution was 17.2 mS cm^{-1}.

At the beginning of each experiment, correct membrane formation was checked by measuring membrane current and capacity. Only membranes which gave rise to a basic current of less than 2.5 pA at a clamp voltage of −80 mV and which had a capacity of more than 90 pF (diameter of the aperture 150 μm) were used for the experiments. The agents were added in aliquots of 15 μl up to the respective final concentration to the compartment opposite to the PL side of the bilayer and the bathing solution was stirred for 30 sec with magnetic bars.

Results

Determination of Molecular Charge and Surface Charge Density

The molecular areas of the various lipids were determined from monolayer isotherms obtained at 37°C at a lateral pressure of 30 mN m^{-1}. For the calculation of the molecular areas and the surface charge densities, the average molecular weights determined mass spectrometrically were used. All values are listed in the Table.

Interaction of PMB with Bilayer Membranes

To study the influence of the composition of the lipid matrix of the bilayer membranes, in particular that of the leaflet to which PMB was added, on the PMB-membrane interaction, various planar membrane systems were pre-

Table. Molecular parameters describing the (glyco)lipids composing the bilayer leaflet at the side of PMB addition of various asymmetric planar bilayers (the second leaflet was always made from the phospholipid mixture) and the average diameters of the induced membrane lesions

(Glyco)lipid	Average molecular mass[a] Da	Molecular area[b,c] nm^2	Molecular charge[b,d] e_0	Charge density[b] e_0/nm^2	Diameter of membrane lesions[b,g,h] nm
F515 LPS	2237	1.3	−4	−3.1	2.4 ± 0.2
F515 LPS (pH 3)	2237	1.2	−2[e]	−1.7	<0.7
R595 LPS	2415	1.4	−3.4	−2.4	2.3 ± 0.5[i]
R45 LPS	2465	1.5	−3.0	−2.0	1.0 ± 0.1
GSL-1	758	0.5	−0.5[f]	−1.0	0.7 ± 0.1
PG	750	0.6	−1	−1.7	<0.5
DPhyPC	846	0.8	0	0	<0.5

[a] Under consideration of chemical heterogeneity as determined by MALDI-TOF mass spectrometry. [b]If not otherwise stated, all data refer to a bathing solution/subphase at pH 7; [c]taken from monolayer isotherms at 37°C and a lateral pressure of 30 mN m^{-1}, SEM ± 5% (an additional error may result from batch-to-batch variations); [d]calculated according to the number of phosphoryl and carboxyl groups under consideration of substitutions; [e]the state of ionization at pH 3 was derived from monolayer and ζ-potential measurements on the PMB binding stoichiometries (*data to be published*); [f]according to (Wiese et al., 1996); [g]at negative clamp voltages; [h]calculated from $I = (\pi \cdot \sigma \cdot d^2 \cdot U)/(4 \cdot 1)$ with I membrane current, σ specific conductivity of the subphase, d diameter of the lesion, U clamp voltage, and 1 membrane thickness; [i]taken from (Schröder et al., 1992).

pared. These differed in one leaflet whereas the other (PL) was the same in all experiments. The interaction was characterized by the amplitudes of the current fluctuations in the initial phase after drug addition and the expression of a macroscopic steady state of conductivity. These parameters were studied in dependence on the applied clamp voltage.

The addition of PMB at a final concentration of 1 μM, which is in the range of the minimal inhibitory concentration (Storm & Rosenthal, 1977), to the LPS side of an asymmetric F515 LPS/PL planar bilayer membrane and at 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 (Fig. 3*A*). Microscopically, the initial phase of the interaction is characterized by short transient current fluctuations (Fig. 3*B*) which are indicative of the induction of transient membrane lesions. Increasing the applied clamp voltages up to −80 mV led to a proportional increase of the amplitudes of the current fluctuations (Fig. 4). The higher clamp voltages caused, however, in many cases a membrane rupture before the steady state was reached.

For a characterization of the fluctuations, their amplitudes and from these the mean current of the single fluctuations were determined (Fig. 5) yielding a value of (−25.7 ± 1.5) pA at a clamp voltage of −20 mV. This value represents the minimum current, because in the current traces (*see also* Figs. 4 and 6) also multiples of this basic value are observed which should result either from larger lesions or from the coincident formation of two or more lesions. From the mean current I of the single fluctuations at a clamp voltage U in a bathing solution of the specific conductance σ, the mean diameter d of a single membrane lesion could be calculated to

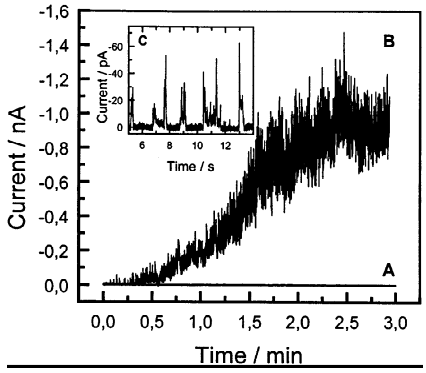


Fig. 3. Macroscopic current traces in the absence (*A*) and presence (*B*) of PMB in the bathing solution. The inlet (*C*) shows details of (*B*) in the initial phase after PMB addition (1 μM PMB added to the LPS side of a F515 LPS/PL bilayer at a clamp voltage of −20 mV). Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

(2.4 ± 0.2) nm according to the relation $I = (\pi \cdot \sigma \cdot d^2 \cdot U)/(4 \cdot 1)$. For this, a circular geometry of the lesion and a membrane thickness 1 of 6 nm was assumed. This value is independent of the applied clamp voltage (*see above*).

To elucidate a possible influence of the lipid molecules forming the leaflet of the bilayer membrane facing the side of PMB addition on the formation of transient membrane lesions, we have varied the lipid composition with respect to the molecular area as well as to the molecular charge of the membrane lipids. Thus, we built this leaflet from LPS of the deep rough mutant isolated from *P. mirabilis* strain R45, from GSL-1 (the glycosphingolipid of *Sphingomonas paucimobilis* IAM 12576 with the shortest sugar moiety — one GlcA attached to the dihydrosphingosine anchor), and from various phospholipids including PG and DPhyPC.

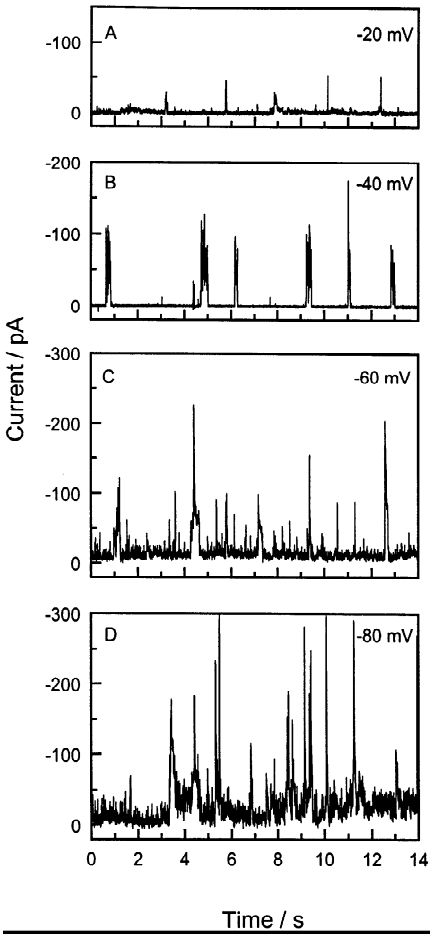


Fig. 4. Current fluctuations in dependence on time in the initial phase after the addition of 1 μM PMB to the LPS side of a F515 LPS/PL bilayer at different clamp voltages. Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

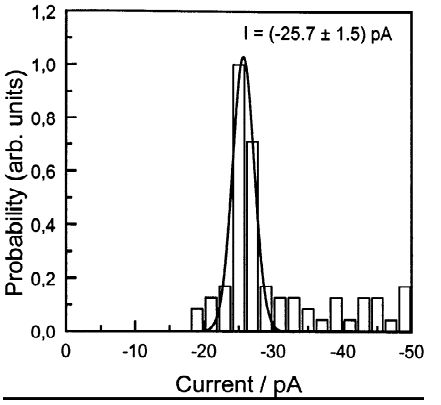


Fig. 5. Distribution of the amplitudes of the current fluctuations induced by 1 μM PMB added to the LPS side of a F515 LPS/PL bilayer at a clamp voltage of −20 mV (bars), fitted by a Gaussian distribution (solid curve). Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

The conductivity responses after PMB addition at a concentration of 1 μM and a clamp voltage of −20 mV were very similar for R595 LPS (Schröder et al., 1992) but significantly different for the other lipids as compared to that described for F515 LPS. The single-channel fluctuations did not occur for GSL-1/PL, PG/PL, and DPhyPC/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 at −20 mV but increased with increasing clamp voltage and resulted in a plateau-like steady state at −80 mV. For PG/PL and DPhyPC/PL membranes a concentration of 50 μM and a clamp voltage of −80 mV were necessary to induce single channel fluctuations and steady states. For obvious reasons, the influence of all possible combinations of PMB concentrations and clamp voltages on these two parameters could not be tested. Nevertheless, it appeared that the macroscopic steady state was observed above a lipid-dependent PMB concentration. Its conductivity was then concentration-independent but depended on the particular lipid matrix. Thus, it was highest for F515 LPS/PL, smaller for R45 LPS/PL, and smallest for GSL-1/PL, PG/PL, and DPhyPC/PL bilayers.

From the conductivities of the single channel fluctuations, the diameters of the membrane lesions were calculated for the different lipid systems. As outlined above, the fluctuations occurred at different threshold clamp voltages characteristic for the differently composed membrane systems. Therefore, the single channel fluctuations were compared at a clamp voltage of −80 mV, which was above the highest threshold value, but at different PMB concentrations (Fig. 6). It should be emphasized once more that the single channel conductivities are voltage-independent and had, thus, no influence on the pore sizes.

As an example, in Fig. 7, 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 respective values for the other lipid matrices are listed in the Table.

The influence of the molecular charge or surface charge density on the formation of membrane lesions through PMB and on their size was studied with F515 LPS/PL bilayers at pH 3 of the bathing solution. This way, the effective molecular charge on the LPS side was reduced, but the molecular geometry was left nearly unchanged (Table). Prior to these experiments we proved that (i) the net charge of PMB did not vary in the pH range from 3 to 8 in titration experiments with NaOH and (ii) the activity of PMB was not influenced by the

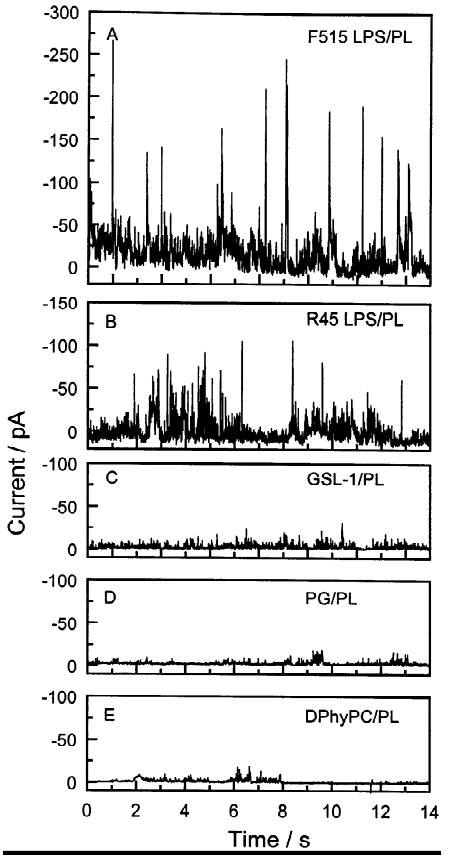


Fig. 6. Current fluctuations in dependence on time in the initial phase after the addition of various concentrations of PMB to asymmetric bilayers at a clamp voltage of −80 mV (the side of PMB addition is named first): (*A*) F515 LPS/PL bilayer, C_{PMB} = 1 μM; (*B*) R45 LPS/PL bilayer, C_{PMB} = 1 μM; (*C*) GSL-1/PL bilayer, C_{PMB} = 15 μM; (*D*) PG/PL bilayer, C_{PMB} = 50 μM; (*E*) DPhyPC/PL bilayer, C_{PMB} = 50 μM. Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

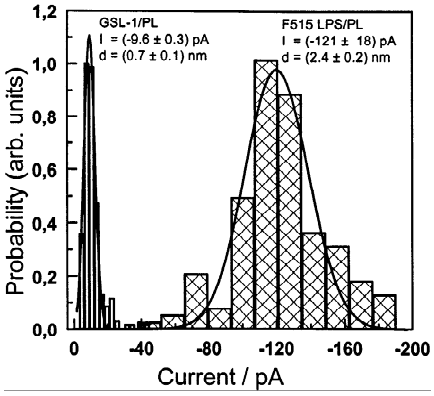


Fig. 7. Distributions of the amplitudes of the current fluctuations induced by the addition of PMB to the glycolipid side of GSL-1/PL and F515 LPS/PL bilayers at a clamp voltage of −80 mV, fitted by Gaussian distributions (solid curves). Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

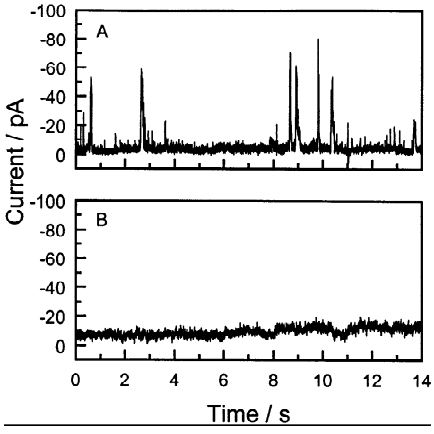


Fig. 8. Current fluctuations in dependence on time in the initial phase after the addition of different concentrations of PMB to the LPS side of a F515 LPS/PL bilayer at a clamp voltage of −40 mV and at different pH. (*A*) C_{PMB} = 1 μM, bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C; (*B*) C_{PMB} = 30 μM, bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 NaCitrate; pH 3, T = 37°C.

low pH by first incubating PMB at pH 3 for 20 min and then adding it to a F515 LPS/PL membrane at pH 7. The induced lesions were in this case identical to those observed under standard conditions (without preincubation). At pH 3, single current fluctuations were observed only above PMB concentrations of 20 μM and clamp voltages of −40 mV (Fig. 8). These fluctuations were very small and even at −80 mV in the range of the current resolution of the apparatus. Thus, only an upper limit of the amplitudes of 10 pA could be determined. Also, a macroscopic state was observed, which was, however, lower than that at pH 7.

Because it is known that the LPS molecules in the outer leaflet are bridged by divalent cations like Mg^{2+} leading to a tight LPS network, it could be assumed that PMB leads to a destabilization of the membrane just by removing the cations. We have performed experiments, in which we treated F515 LPS/PL membranes with Tris-EDTA (5–10 mM) to test this assumption, and no membrane destabilization was observed. Furthermore, PMB induced very similar membrane lesions in the absence or presence of Mg^{2+} in the bathing solution (*data not shown*).

Besides evaluating the two parameters, the amplitudes of the single current fluctuations and the macroscopic steady state, for the various experimental conditions, we have also qualitatively compared the lifetimes of the single current fluctuations from Fig. 6. We found that the lifetimes were longest for F515 LPS/PL membranes (up to 100 msec), shorter for R45 LPS/PL mem-

branes (some msec), and shortest for the other membrane systems (around 1 msec).

Interaction of Detergents with Bilayer Membranes

We have previously proposed a detergent-like behavior of PMB. Detergents are known to form micelles at relatively high concentrations (typically in the millimolar range in pure aqueous environments). In light scattering experiments we could show that PMB formed aggregates, not necessarily micelles, at concentrations around 10 mM. It may be assumed that for the formation of membrane lesions a high local concentration of the inducing agent is a prerequisite. We have, therefore, compared the lesion-inducing capacity of the polycationic PMB and some detergents including positively charged (DTPPB and DTMAB) and negatively charged (SDS). The CAC of the detergents have been determined in light scattering experiments or were taken from the literature when available. They are 1.49 mM for SDS in 0.1 M NaCl and 14 mM for DTMAB in distilled water both at 35°C (Mukerjee & Mysels, 1970) and 1 mM for DTPPB in an aqueous phase corresponding the bathing solution. The lower CAC value of DTPPB may be explained by the higher lipophilicity (π-value) of phenyl as compared to that of methyl groups (Seydel & Schaper, 1979). It should be noted that in the presence of membranes these values may be significantly lower. The effect of the classic detergents were investigated only with F515 LPS/PL membranes.

In Fig. 9, the current traces obtained from F515 LPS/PL membranes after the addition of 0.1 mM of DTMAB (A) or SDS (B), respectively, at a clamp voltage of −80 mV are shown. Obviously, the current fluctuations induced by the two detergents are very similar to those induced by PMB. It must be pointed out, however, that the amplitudes of the fluctuations were significantly higher in the case of PMB (*see* Fig. 4*D*). Furthermore, whereas in the presence of DTMAB also a macroscopic steady state was obtained, the membranes tended to break in the presence of SDS before a steady state was reached. Using DTPPB instead of DTMAB, current fluctuations could be observed already at a tenfold lower concentration (*data not shown*).

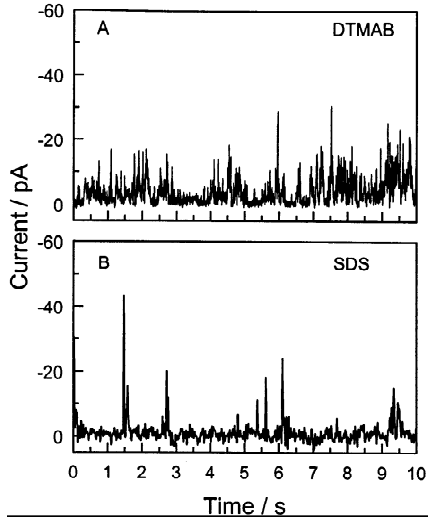


Fig. 9. Current fluctuations in dependence on time in the initial phase after the addition of 0.1 mM DTMAB (*A*) and 0.1 mM SDS (*B*) to the LPS side of a F515 LPS/PL bilayer at a clamp voltage of −80 mV. Bathing solution (in mM): 100 KCl, 5 $MgCl_2$, 5 HEPES; pH 7, T = 37°C.

Discussion

In earlier investigations into the interaction of PMB with the reconstituted lipid matrix of the outer membrane (Schröder et al., 1992), we observed transient current fluctuations with short lifetimes (up to 100 msec) immediately after drug addition to the LPS side of a R595 LPS/PL bilayer, which superimposed and terminated in a macroscopic steady state. We found that the diameters of underlying membrane lesions were large enough to allow single PMB molecules to permeate the bilayer (self-promoted transport). From these findings, we proposed a model for the PMB action which was based on a detergent-like action of PMB. According to this model, the lamellar bilayer structure would be disturbed by nonlamellar structures formed either by PMB molecules alone or by a mixture of PMB and lipid molecules.

From a comparison of Fig. 3 showing the current response to the interaction of PMB with F515 LPS/PL bilayers with respective data on R595 LPS/PL membranes (Schröder et al., 1992) it can be taken that these two membrane systems behave very similarly upon the action of the drug. This should not be surprising in view of the fact that the two LPS have nearly identical structures and differ only slightly in the effective molecular charge (Table).

To further elucide the open questions to the mechanisms underlying the formation of membrane lesions, in particular to the influence of the lipid matrix, in the present investigations we characterized the interaction of PMB with various bilayer membrane systems. In these bilayers, one leaflet was left unchanged (PL mixture) and the other (to which the peptide was added) was varied. The action of PMB was characterized by evaluating the amplitudes of the induced current fluctuations and the expression of a macroscopic steady state of membrane current. These experiments should also provide insight into the mechanisms responsible for PMB resistance, even though we must point out once more that for the reconstitution experiments of the outer membrane only

Re LPS and GSL with the shortest sugar moiety were used for reasons of membrane stability. To obtain additional information on the proposed detergent-like mechanism of action of PMB, we performed experiments on the action of classical anionic and cationic detergents on F515 LPS/PL bilayers.

In contrast to the early work by Miller et al. (1978), we detected the PMB-induced formation of transient membrane lesions. We found that for this formation to occur the composition of the lipid matrix of the bilayer membrane plays an essential role. Thus, the PMB concentration as well as the clamp voltage above which the transient current fluctuations could be observed were significantly lower for F515 LPS/PL than for all other membrane systems investigated (Fig. 6). Also, in agreement with earlier data on R595 LPS/PL membranes (Schröder et al., 1992), the mean current of the membrane lesions was proportional to the applied clamp voltage in the range of investigated voltages (Fig. 4). Furthermore, the diameters of the PMB-induced lesions were matrix-dependent and could be arranged in the order F515 LPS/PL, R595 LPS/PL > R45 LPS/PL > GSL-1/PL > PG/PL > DPhyPC/PL membranes (Fig. 6 and Table). It should be noticed, however, that the lesions induced in F515 LPS/PL membranes at pH 3 of the bathing solution were significantly smaller than at pH 7 and comparable to those induced in PG/PL membranes (Figs. 6, 8, and Table).

Our experiments on the action of PMB in the absence and presence of Mg^{2+} ions in the bathing solution using F515 LPS/PL membranes and with membranes treated with Tris-EDTA provided strong evidence, that the action of PMB goes beyond a mere destabilization of the bilayer via the displacement of divalent cation bridges. This mechanism was discussed, for example, by Hancock (1984) and Vaara (1992).

Already Teuber and Bader (1976) 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 finding, we could demonstrate an obviously good correlation between the diameter of the lesions and the molecular charge and the surface charge density, but not the molecular area (Table). Since the various surface lipids in our reconstitution experiments carry different numbers of net negative charges and PMB carries five positive charges, a correlation between the binding stoichiometry of PMB and the various membrane lipids could also be expected. A definite decision as to which of these parameters is the governing one is not possible on the basis of the available data, because a change of the molecular charge by keeping the molecular geometry unchanged — achieved with F515 LPS on the one hand (four negative charges) and R45 LPS (3 negative charges) and F515 LPS at pH 3 (2 negative charges) on the other hand while the net charge of PMB is identical at pH 7 and pH 3 — does not only reduce the surface charge density but most likely also the LPS-PMB binding stoichiometry. Furthermore, it has to be considered that the determination of the molecular charge and the surface charge density is subject to uncertainties because only limited data on the state of ionization of the dissociable phosphoryl, carboxyl and amine groups of the lipids within a membrane surrounded by a diffuse double layer have been published. According to data summarized by J.-F. Tocanne and J. Teissié (1990), the net charge of PG and phosphatidylcholine should — under our experimental conditions — be 1 and 0, respectively. Din et al. (1993) concluded from solubility measurements at near physiological pH of 7.4 that Re LPS isolated from *E. coli* D31m4, which is structurally identical to F515 LPS, in aggregated form is predominantly tetraanionic, containing two monoanionic phosphates and completely dissociated Kdo's. For GSL-1-aggregates we know from earlier FTIR-measurements (Wiese et al., 1996) that the carboxyl group of the glucuronic acid carries on the average only 0.5 negative charges at physiological pH. In any case, it may be proposed that a sufficiently high concentration of PMB molecules at a locally restricted area of the membrane surface is a prerequisite for the formation of membrane lesions. This is achieved when PMB clusters interact with the membrane. The formation of PMB clusters may occur under different conditions: (i) electrostatic attraction leads to an accumulation of positively charged PMB molecules at negatively charged membrane surfaces in dependence on the surface charge density to a concentration higher than the CAC, (ii) the PMB concentration in the aqueous phase is *a priori* higher than the CAC, or (iii) density fluctuations in the bathing solution lead to the cluster formation even below the CAC (Israelachvili, 1991). The insertion of PMB clusters, which is enhanced by electrostatic interaction, leads to the formation of locally restricted lipid-PMB complexes of different stoichiometries depending on the molecular charge. These complexes exert a local stress in the lamellar bilayer, which finally causes the formation of membrane lesions. The lesions are the larger the more PMB molecules are bound per lipid molecule. Multiples of the basic values of the current fluctuations are observed (Figs. 4 and 6) when the local PMB concentration is high enough for the formation of more than one membrane lesion in a locally restricted area. The thus formed lesions are not statistically independent, whereas lesions formed coincidently at different areas of the membrane would be independent, however, the latter processes are very unlikely and cannot explain the high number of multiples.

The limited lifetimes of the induced lesions may be explained by the lateral diffusion of the PMB molecules in the membrane due to concentration gradients leading

to a decrease in the local PMB concentration below the critical value required for the formation of nonlamellar structures. Also, the macroscopic steady state (Fig. 3) can be explained by the lateral diffusion leading to an equilibrium concentration of PMB in the membrane. Thus, the further addition of PMB after the steady state is reached does not lead to a further increase in membrane current (Schröder et al., 1992).

Differences in the lifetimes of the lesions observed in the various lipid matrices (up to 100 msec for F515 LPS/PL and R595 LPS/PL (Schröder et al., 1992), some ms for R45 LPS/PL, or about 1 msec for the other bilayers, Fig. 6) may be caused by different diffusion rates of the PMB molecules in the various lipid environments and/or different qualities of the binding of PMB to the various lipid species due to the stoichiometrically and geometrically determined arrangement of the PMB and lipid molecules.

The interaction of PMB with DPhyPC surfaces may be surprising. However, for an interpretation of the observed effects it must be considered that the PMB concentrations required for the induction of membrane lesions was comparatively high, that the amplitudes of the current fluctuations were comparatively low, and that PMB is supposed to act like a detergent. This means that at sufficiently high concentrations PMB could also interact with neutral surfaces as does the negatively charged SDS with negatively charged surfaces (Fig. 9*B*), pointing at the involvement of hydrophobic forces in the interaction process. Lastly, the inner molecular charge distribution of DPhyPC — the negative charge of the phosphate group is compensated by the positive charge of the choline group — should not be neglected.

The PMB molecule is too large to permeate the outer membrane through the porin channels to reach its locus of action, the cytoplasmic membrane (Vaara, 1992). It has rather to penetrate the lipid matrix of the outer membrane. In this context, the self-promoted uptake has been discussed (Hancock, 1984) and, therefore, the PMB-induced transient membrane lesions attain importance as pathways for facilitated diffusion. 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 obviously fulfilled. The diameters of the lesions are about 2.4 nm (Figs. 5, 7, and Table), whereas the cross section of the PMB molecule is 1.23 nm (El Mashak & Tocanne, 1980). Of course, the lesions allow also other small molecules and ions to permeate the outer membrane in both directions. The rough mutants *E. coli* strain F515 and *S. enterica* sv. Minnesota strain R595 are known to be 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 (Fig. 7 and Table). The respective Gram-negative species are known to be PMB-resistant (Kaca, Radziejewska-Lebrecht & Bhat, 1990; Yabuuchi et al., 1979).

It is known that the 4′-phosphate of the lipid A and the first Kdo of the LPS of the *a priori* PMB-resistant strain *P. mirabilis* R45 are substituted by cationic L-Ara*p*4N (Kaca et al., 1990). Also, it could be shown that in the PMB-resistant mutants of *E. coli*, *S. enterica* sv. Typhimurium, and *S. marcescens*, L-Ara*p*4N and 2-aminoethanol were present in lipid A, whereas these substituents were absent in the PMB-sensitive strains (Vaara et al., 1981; Nummila et al., 1995; Seltmann et al., 1996). These substitutions provoke a reduction on the molecular charge and the surface charge density (changes in the molecular area can be neglected). 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 the bound PMB molecules and with that to a reduction in the size of the transient lesions. The importance of the molecular charge for the PMB activity is further emphasized by the respective data on GSL-1/PL membranes. The GSL-1 molecule carries *per se* only one half negative charge, and the PMB-induced lesions are extremely small.

The importance of the macroscopic steady states of the membrane current as a parameter describing the PMB resistance or sensitivity can at present not be judged. Obviously, there is a direct correlation between the occurrence and the amplitudes of the PMB-induced current fluctuations and the expression and the height of the macroscopic steady state of the membrane current: only in those cases where current fluctuations are observed, a steady state is expressed with its height correlating with the amplitudes of the fluctuations.

We have previously proposed a detergent-like action for PMB (Schröder et al., 1992). To further support this model, we have investigated the interaction between the classical detergents DTMAB, DTPPB, and SDS and F515 LPS/PL membranes. All detergents induced current fluctuations similar to those observed with PMB (Fig. 9). However, the minimal concentrations required for the formation of the transient lesions depended on the particular detergent. For the positively charged compounds, the concentrations correlated roughly with their CAC values: the minimal concentration of the more lipophilic DTPPB was 10 μM and with that tenfold lower than that of DTMAB. The minimal concentration for the anionic SDS was 0.1 mM. Thus, the minimal concentrations of the detergents for the induction of transient lesions correlate qualitatively with their CAC, in particular when considering that the positively charged compounds should be accumulated at the membrane surface and the

negatively charged SDS rejected. A quantitative agreement is hardly to be achieved, because this would require a large number of experiments and the knowledge of the effective concentration at the membrane surface. Also in this context it should be considered that even below the CAC aggregates may be formed due to concentration fluctuations. Thus, the concentration-dependent induction of current fluctuations by the detergents seems once more to support the hypothesis that sufficiently high local concentrations of the agents at the membrane surface are a prerequisite for the formation of transient membrane lesions.

For the significantly lower amplitudes of the detergent-induced fluctuations than those induced by PMB in the respective lipid bilayer (Figs. 6*A* and 9) we can, at present, give no well-founded explanation. We may only speculate that this phenomenon is due to the smaller size of the detergent molecules and/or to a smaller ratio of the cross sectional areas of the hydrophilic and the hydrophobic moieties.

Our data clearly support the importance of a self-promoted transport process for PMB molecules as a prerequisite for its activity. This is derived from the observation that in reconstituted outer membranes of PMB-sensitive strains the PMB-induced membrane lesions are large enough, whereas in membranes of strains with known PMB-resistance they are too small to allow PMB molecules to permeate. The data, furthermore, show that for the initiation of the formation of single membrane lesions a threshold concentration of the drug at locally restricted areas at the membrane surface is required and that the lipid molecules of the bilayer matrix are involved in the formation of the lesions. Finally, strong evidence in support of the proposed detergent-like action of PMB could be provided by showing that various classical detergents induce similar phenomena in the reconstituted lipid matrix of the outer membrane. Beyond providing insight into the mechanisms of PMB-membrane interactions, this work points out the capability of the applied techniques for the elucidation of peptide/protein-membrane interactions in general.

We are indebted to Mr. D. Koch for performing the film balance measurements, to Mrs. H. Lüthje for the MALDI TOF mass spectrometric analyses, and to Mrs. M. Lohs for the graphical work. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 470, project B5) and the Federal Minister of Education, Science, Research, and Technology (BMBF-grant 01 KI 9471, project A6)

References

Benz, R., Bauer, K. 1988. Permeation of hydrophilic molecules through the outer membrane of gram-negative bacteria. Review on bacterial porins. *Eur. J. Biochem.* **176:**1–19

Blume, A. 1979. A comparative study of the phase transition of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta* **557:**32–44

Bucklin, S.E., Lake, P., Logdberg, L., Morrison, D.C. 1995. Therapeutic efficacy of a polymyxin B-dextran 70 conjugate in experimental model of endotoxemia. *Antimicrob. Agents Chemother.* **39:**1462–1466

Din, Z.Z., Mukerjee, P., Kastowsky, M., Takayama, K. 1993. Effect of pH on solubility and ionic state of lipopolysaccharide obtained from the deep rough mutant of *Escherichia coli*. *Biochemistry* **32:**4579–4586

Ebara, Y., Okahata, Y. 1994. A kinetic study of concanavalin A binding to glycolipid monolayers by using a quartz-crystal microbalance. *J. Am. Chem. Soc.* **116:**11209–11215

El Mashak, E.M., Tocanne, J.F. 1980. Polymyxin B-phosphatidylglycerol interactions A monolayer (π, ΔV) study. *Biochim. Biophys. Acta* **596:**165–179

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

Gough, M., Hancock, R.E.W., Kelly, N.M. 1996. Antiendotoxin activity of cationic peptide antimicrobial agents. *Infect. Immun.* **64:**4922–4927

Hancock, R.E.W. 1984. Alterations in outer membrane permeability. *Ann. Rev. Microbiol.* **38:**237–264

Helander, I.M., Kato, Y., Kilpeläinen, I., Kostiainen, R., Lindner, B., Nummila, K., Sugiyama, T., Yokochi, T. 1996. Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O3. *Eur. J. Biochem.* **237:**272–278

Hirata, M., Zhong, J., Wright, S.C., Larrick, J.W. 1995. Structure and functions of endotoxin-binding peptides derived from CAP18. *Prog. Clin. Biol. Res.* **392:**317–326

Israelachvili, J.N. 1991. Thermodynamic principles of self-assembly. *In:* Intermolecular & Surface Forces. pp. 341–365. Academic Press, London, San Diego, New York, Boston, Sydney, Tokyo, Toronto

Kaca, W., Radziejewska-Lebrecht, J., Bhat, U.R. 1990. Effect of polymyxins on the lipopolysaccharide-defective mutants of *Proteus mirabilis*. *Microbios.* **61:**23–32

Kawahara, K., Seydel, U., Matsuura, M., Danbara, H., Rietschel, E.T., Zähringer, U. 1991. Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. *FEBS Lett.* **292:**107–110

Levy, O., Ooi, C.E., Elsbach, P., Doerfler, M.E., Lehrer, R.I., Weiss, J. 1995. Antibacterial proteins of granulocytes differ in interaction with endotoxin. Comparison of bactericidal/permeability-increasing protein, p15s, and defensins. *J. Immunol.* **154:**5403–5410

Marcelja, S. 1974. Chain ordering in liquid crystals. II. Structure of bilayer membranes. *Biochim. Biophys. Acta* **367:**165–176

Miller, I.R., Bach, D., Teuber, M. 1978. Effect of polymyxin B on the structure and the stability of lipid layers. *J. Membrane Biol.* **39:** 49–56

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Mukerjee, P., Mysels, K.J. 1970. *In:* Criticall Micelle Concentrations of Aqueous Surfactant Systems. Secretary of Commerce on Behalf of the US Government, Washington, D.C.

Nagle, J.F. 1976. Theory of lipid monolayer and bilayer phase transitions: effect of headgroup interactions. *J. Membrane Biol.* **27:**233–250

Nakae, T. 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. *Biochem. Biophys. Res. Commun.* **71:**877–884

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

Nummila, K., Kilpeläinen, I., Zähringer, U., Vaara, M., Helander, I.M. 1995. Lipopolysaccharides of polymyxin B-resistant mutants of

Escherichia coli are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol. Microbiol.* **16:**271–278

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

Ried, C., Wahl, C., Miethke, T., Wellnhofer, G., Landgraf, C., Schneider-Mergener, J., Hoess, A. 1996. High affinity endotoxin-binding and neutralizing peptides based on the crystal structure of recombinant Limulus anti-lipopolysaccharide factor. *J. Biol. Chem.* **271:**28120–28127

Rietschel, E.T., Brade, L., Lindner, B., Zähringer, U. 1992. Biochemistry of lipopolysaccharides. *In:* Bacterial Endotoxic Lipopolysaccharides, Vol. I: Molecular biochemistry and cellular biology. D.C. Morrison and J.L. Ryan, editors. pp. 3–41. CRC Press, Boca Raton

Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A.J., Zähringer, U., Seydel, U., Di Padova, F., Schreier, M., Brade, H. 1994. Bacterial endotoxin: Molecular relationships of structure to activity and function. *FASEB J.* **8:**217–225

Schröder, G., Brandenburg, K., Seydel, U. 1992. Polymyxin B induces transient permeability fluctuations in asymmetric planar lipopolysaccharide/phospholipid bilayers. *Biochemistry* **31:**631–638

Seltmann, G., Lindner, B., Holst, O. 1996. Resistance of *Serratia marcescens* to polymyxin B: a comparative investigation of two S-form lipopolysaccharides obtained from a sensitive and a resistant variant of strain 111. *J. Endotoxin Res.* **3:**497–504

Seydel, J.K., Schaper, K.-J. 1979. *In:* Chemische Struktur und Biologische Aktivität von Wirkstoffen. Verlag Chemie, Weinheim, New York

Seydel, U., Schröder, G., Brandenburg, K. 1989. Reconstruction of the lipid matrix of the outer membrane of Gram-negative bacteria as asymmetric planar bilayer. *J. Membrane Biol.* **109:**95–103

Srimal, S., Surolia, N., Balasubramanian, S., Surolia, A. 1996. Titration calorimetric studies to elucidate the specificity of the interactions of polymyxin B with lipopolysaccharides and lipid A. *Biochem. J.* **315:**679–686

Storm, D.R., Rosenthal, K. 1977. Polymyxin and related peptide antibiotics. *Ann. Rev. Biochem.* **46:**723–763

Teuber, M., Bader, J. 1976. Action of polymyxin B on bacterial membranes. Binding capacities for polymyxin B of inner and outer membranes isolated from *Salmonella typhimurium* G30. *Arch. Microbiol.* **109:**51–58

Thelestam, M., Mollby, R. 1980. Cultured human fibroblasts as a model for evaluation of potential in vivo toxicity of membrane damaging antibiotics. *Chemical and Biological Interactions* **29:**315–325

Tocanne, J.F., Teissié, J. 1990. Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion of protons in membrane model systems. *Biochim. Biophys. Acta* **1031:**111–142

Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurminen, M., Rietschel, E.T., Mäkelä, P.H. 1981. Characterization of the lipopolysaccharide from the polymyxin-resitant *pmrA* mutants of *Salmonella typhimurium. FEBS Lett.* **129:**145–149

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56:**395–411

Vaara, M., Porro, M. 1996. Group of peptides that act synergistically with hydrophobic antibiotics against gram-negative enteric bacteria. *Antimicrob. Agents Chemother.* **40:**1801–1805

Vinogradov, E.V., Thomas-Oates, J.E., Brade, H., Holst, O. 1994. Structural investigation of the lipopolysaccharide from *Proteus mirabilis* R45 (Re-chemotype). *J. Endotoxin Res.* **1:**199–206

Wiese, A., Reiners, J.O., Brandenburg, K., Kawahara, K., Zähringer, U., Seydel, U. 1996. Planar asymmetric lipid bilayers of glycosphingolipid or lipopolysaccharide on one side and phospholipids on the other: Membrane potential, porin function, and complement activation. *Biophys. J.* **70:**321–329

Yabuuchi, E., Tanimura, E., Ohyama, A., Yano, I., Yamamoto, A. 1979. *Flavobacterium devorans* ATCC 10829: A strain of *Pseudomonas paucimobilis. J. Gen. Appl. Microbiol.* **25:**95–107

Zähringer, U., Lindner, B., Seydel, U., Rietschel, E.T., Naoki, H., Unger, F.M., Imoto, M., Kusumoto, S., Shiba, T. 1985. Structure of de-O-acylated lipopolysaccharide from the *Escherichia coli* Remutant strain F 515. *Tetrahedron Letters* **26:**6321–6324