J. Membrane Biol. 48, 365–383 (1979)

Asymmetry of the Gramicidin Channel in Bilayers of Asymmetric Lipid Composition: I. Single Channel Conductance

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Received 15 December 1978; revised 13 March 1979

Summary. Gramicidin-doped asymmetric bilayers made by the Montal-Mueller method exhibited an asymmetric current-voltage relationship. The asymmetric conductance was shown to be the product of two components, a rectifying single-channel conductance and an asymmetric voltage dependence of the reaction which leads to the conducting channel. The single-channel conductance was asymmetric in both asymmetric bilayers made of charged lipids and asymmetric bilayers made only of neutral lipids. The single-channel asymmetry decreased with increasing ion concentration. From the comparison of the single-channel conductance in symmetric and asymmetric bilayers and the dependence of the asymmetry on the solution ion concentrations, it was concluded that (1) the rate of ion entry into the channel is dependent on the lipid composition of the membrane and is asymmetric in asymmetric bilayers; (2) the entry step is rate determining at low ion concentrations; and (3) at higher ion concentrations the rate-determining step is the translocation across the main barrier in the membrane; and this translocation appears insensitive to lipid asymmetry.

The linear pentadecapeptide gramicidin has been increasingly studied as a model substance for ion channels in biological membranes. Gramicidin's properties as an ion channel can be deduced if controlled amounts are incorporated into artificial bilayer membranes and macroscopic as well as single channel conductance measurements are made. The single-channel conductance saturates at high salt concentrations and in some cases even decreases at still higher concentrations (Hladky, 1974), and gramicidin thus has some electrical properties similar to the Na channel of the nerve membrane (Hille, 1975; Chizmadjev & Aityan, 1977). Since an artificial bilayer containing gramicidin can be controlled in more

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0022-2631/79/0048-0365 $03.80
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ways than a nerve membrane, a greater variety of experimental conditions can be exploited to test predictions from mathematical models of ion conductance through the channel.

Several models have been developed to describe the mechanism of ion transport through the gramicidin pore (Läuger, 1973; Hladky, Urban & Haydon, 1978; Sandblom, Eisenman & Neher, 1977; Levitt, 1978; Eisenman, Sandblom & Neher, 1978). All use Eyring rate theory to describe how the ions cross a series of energy barriers and troughs on their way through the pore. They differ in detail as to the maximum number of ions allowed in the channel at a given time, the number of ion binding sites near the mouth of the channel, and the number of energy barriers encountered by the ion in the channel during its translocation through the membrane (*see* Eisenman *et al.*, 1978, for a comparison of some of these models).

All previous electrical measurements of the gramicidin channel have been performed in symmetrical membranes (bilayers with the same lipid or lipid mixture in both membrane halves). This is a simplifying experimental condition, and all mathematical models of the gramicidin conductance have implicitly made use of the assumption that the channel is symmetrical with respect to the plane separating the two membrane monolayers. Although this assumption has been challenged and a potentially asymmetric (structural but not kinetic) model of the gramicidin pore has been proposed (Veatch & Blout, 1974; Veatch, Fossel & Blout, 1974), recent evidence suggests that the pore is created by a voltage-dependent dimerization of two monomers (Bamberg & Läuger, 1973) and is the product of a head-to-head association (Apell *et al.*, 1977; Bamberg, Apell & Alpes, 1977; Bradley *et al.*, 1978), thus creating a symmetrical channel.

Biological channels, however, are not necessarily symmetric. It is known that the two halves of many biological membranes have different lipid composition, the erythrocyte membrane being the best studied (Bretscher, 1973; Zwaal *et al.*, 1975). In the same way, biological membranes have different peripheral proteins on the two surfaces, and intrinsic membrane proteins spanning the membrane are asymmetrically inserted. Therefore, it is important to have a simple experimental channel system with known asymmetry to study how the channel properties are affected by an asymmetric environment.

We have employed the method of Montal and Mueller (Montal & Mueller, 1972; Benz *et al.*, 1975) to form asymmetric bilayers by adjoining two lipid monolayers of different composition. It has been demonstrated

with the use of carrier antibiotics that this technique can produce asymmetric bilayers. Hall and Latorre (Hall & Latorre, 1976; Latorre & Hall, 1976) showed that the current-voltage curve of the nonactin-induced conductance of these bilayers was asymmetric and that the asymmetry could be quantitatively correlated with the membrane surface potential due to surface charges or the dipole moment of the component lipids. In the present report, the asymmetry of the gramicidin-induced bilayer conductance is additional proof for the asymmetric nature of bilayers made by the Montal-Mueller method with two different lipids. We are more concerned, however, with using alterations in the composition of the two halves of the bilayer to investigate ion transport through the gramicidin channel.

Since gramicidin shows different single channel conductances as well as different kinetics of channel formation in membranes of different lipids (Bamberg *et al.*, 1976; Kolb & Bamberg, 1977; Neher & Eibl, 1977), it may be expected that in asymmetric membranes, gramicidin will behave asymmetrically. In this paper we report on the asymmetry of the single channel conductance, and in an accompanying paper (Fröhlich, 1979) we report that the dimerization step also is asymmetric in its voltage dependence in these asymmetric bilayers. We do not, however, attempt yet to fit the data quantitatively to a new or a published model, but rather add them to the list of observations which will have to be considered in a full description of the conductance mechanism of the gramicidin channel. A preliminary report of these findings has been published previously (Fröhlich, 1978).

Materials and Methods

Gramicidin was purchased from Nutritional Biochemicals (Cleveland, Ohio) and employed without further purification. It was added to the aqueous phase from a methanolic stock solution which was stored in the freezer. PE[1] and PS[1] from beef brain were isolated by K. Janko according to Sanders (1967). K. Janko also synthesized dioleoyl-PS, dierucoyl-PC and dioleoyl-PE (Benz & Janko, 1976). GMO and GME were obtained from Nu Check Prep (Elysian, Minn.). The lipids were chromatographically pure, and the brain PE and PS were repeatedly checked after a month storage to ensure that the purity was maintained. All other reagents were analytical grade.

Bilayer membranes were formed according to the technique of Montal and Mueller (Montal & Mueller, 1972; Benz *et al.*, 1975). The hole in the Teflon septum was made

[1] The following abbreviations are used: PC – phosphatidyl choline; PC 18:1 – dioleoyl-PC; PC 22:1 – dierucoyl-PC; PE – phosphatidyl ethanolamine; PE 18:1 – dioleoyl-PE; PS – phosphatidyl serine; GMO – glyceryl monooleate; GME – glyceryl monoerucate.

by approaching it with a hot platinum wire, thus melting the material away from the heated zone. The diameter of the hole was 0.2–0.3 mm. The septum was clamped between two Teflon troughs each with a 2-ml capacity, thus separating the aqueous phase into two compartments. When assembled, a small amount (5–10 μl) of a vaseline solution in pentane was dropped onto both sides of the septum to cover an area around the hole in the septum which was approximately ten times the size of the hole. The thin film of vaseline which spanned the hole after evaporation of the pentane was removed by a glass fiber. For more procedural details, *see* Benz *et al.* (1975). The experiments were performed at room temperature.

Membrane formation was monitored by the rise of the membrane capacitance charging current and membrane conductance. A triangular voltage pulse was applied via a pair of Ag/AgCl electrodes (with or without KCl-agar bridges) in the aqueous phase. In the macroscopic conductance measurements the current was directly displayed on the screen of a Tektronix 5103 oscilloscope in the *x-y* mode (current on the *y*, voltage on the *x*-axis) by the voltage drop it produced across the oscilloscope input, or it was amplified with a Keithley 427 current amplifier and plotted on a *x-y* recorder (Hewlett-Packard, model 7000 AM) or displayed on the oscilloscope screen. In the single channel experiments, a dc voltage was applied from a battery and voltage divider and the amplified current was stored on magnetic tape (model 6200, Precision Instruments) and played back at slower speed for recording on a strip chart recorder.

As will be seen later, gramicidin is a sensitive probe of membrane asymmetry. It was, therefore, very important to ensure that an observed asymmetric current-voltage curve in fact reflected the intrinsic membrane lipid asymmetry and was not due to an artifact caused by the membrane torus, bulging of the membrane, orientation of the Teflon septum, residual pentane solvent, etc. All of these factors could lead to artifacts, and under certain conditions did induce an asymmetry even in bilayers of symmetric lipid distribution, as observed in an asymmetric current-voltage curve. Therefore, care was taken to minimize any artifactual contribution to the membrane asymmetry, and all bilayer systems were checked for their asymmetry in all possible variations as to the orientation of the Teflon septum, the troughs, electrodes, and the order of steps taken to form the bilayer. The asymmetries reported here were reproducible in all these configurations.

Results

The stationary gramicidin conductance in an asymmetric bilayer consisting of one monolayer of PS and one monolayer of PC 22:1 in 10 mM KCl is the slope of the current-voltage relationship shown in Fig. 1. It is strongly asymmetric, an indication of the asymmetry of this bilayer. The magnitude of the asymmetry persisted for at least 20 hr. If during formation the lipids in the bilayer were accidentally mixed due to repeated making and breaking of membranes, the initially low conductance asymmetry returned to the same final value after a few hours. This is evidence that at least in this bilayer the rate of flip-flop of the individual lipid molecules between the two halves of the bilayer was small compared to the rate at which the lipids of each membrane half were exchanged

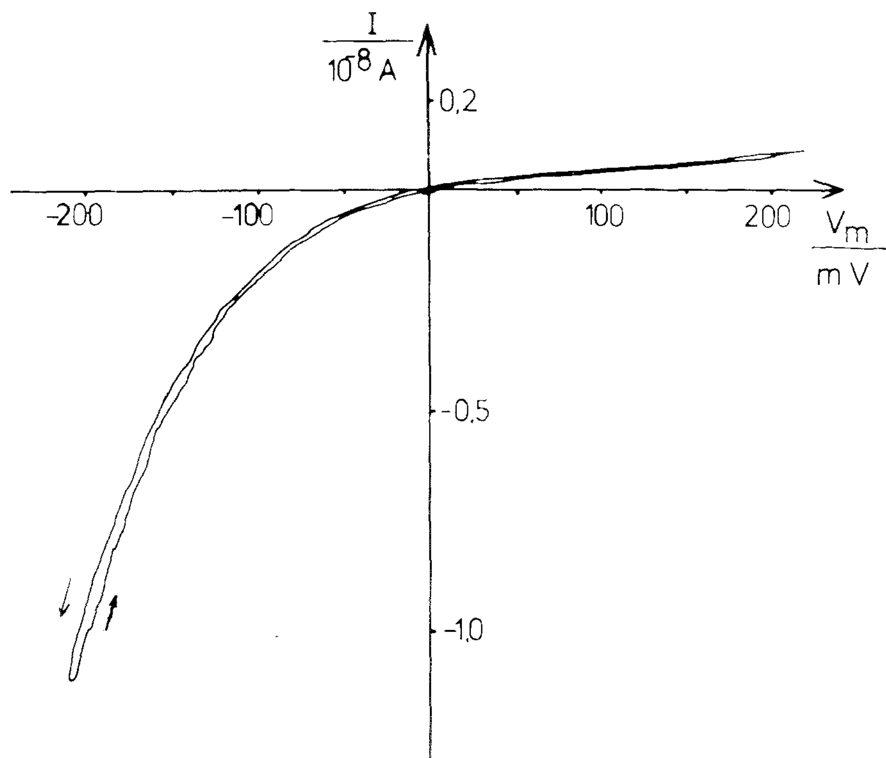


Fig. 1. Quasi-stationary current-voltage curve of gramicidin conductance in an asymmetric PS/PC 22:1 bilayer. It was obtained by applying a triangular voltage pulse of 0.01 Hz frequency. The positive current corresponds to the flux of cations from the PC side to the PS side of the membrane. Hysteresis is due to residual conductance relaxations because of insufficiently slow sweep rate. Symmetrical aqueous solution contained 10 mM KCl

with the respective lipid pools surrounding the bilayer by lateral diffusion (Sherwood & Montal, 1975; Hall & Latorre, 1976)[2].

The current-voltage curve in Fig. 1 shows a slight hysteresis which stems from the experimental conditions employed. Since the macroscopic gramicidin conductance has a voltage-dependent dimerization step (Bamberg & Läuger, 1973), the frequency of the triangular voltage pulse must be slow enough to ensure that the number of conducting and nonconducting channels are in equilibrium at each membrane potential. In the case of Fig. 1, the sweep frequency was not sufficiently slow so that a residual relaxation is observed as hysteresis. Because of the voltage-dependence of channel formation, the stationary conductance

[2] This is not the case in all asymmetric bilayer systems: a bilayer of GMO and PS loses its asymmetry within 1 hr as probed by gramicidin, valinomycin and nonactin (*unpublished result*), similar to the observation of Sherwood and Montal (1975).

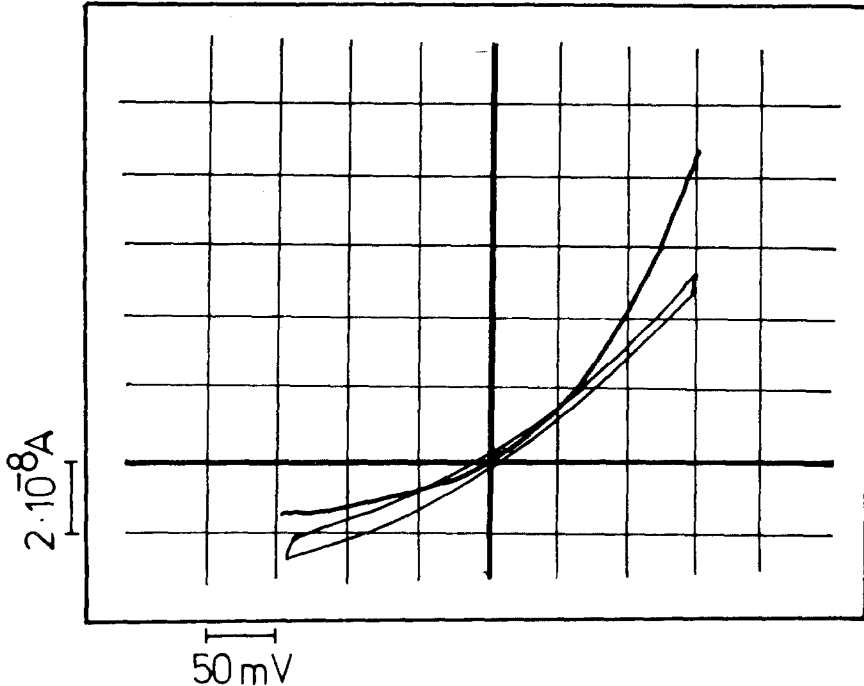


Fig. 2. Current-voltage curves of an asymmetric brain-PE/PS bilayer obtained by applying triangular voltage pulses of different frequency. The stationary conductance curve (single line) was obtained at a sweep rate of 0.01 Hz, and the instantaneous conductance (hysteresis curve) at 20 Hz. Hysteresis in this case was due to membrane capacitance charging current. $I=0$ and $V=0$ are indicated by thick lines of grid. Positive current corresponds to flux of cations from PS to PE side. Symmetrical aqueous solution contained 100 mM NaCl

does not represent the single channel conductance alone. The two components can be separated, however, by sweeping the membrane potential at a frequency which is high compared with the mean lifetime of an open gramicidin channel. At sufficiently high frequencies, the instantaneous conductance is measured. Since the number of open channels does not change during the entire sweep cycle at high frequency, the shape of this instantaneous macroscopic conductance is the same as the shape of the single channel conductance. (Instantaneous current-voltage curves were obtained with other methods and used to derive the results of Fig. 4*b* below.) To prove this, we measured both this instantaneous macroscopic conductance and the single channel conductance in PE/PS asymmetric membranes (Figs. 2 and 3). The only differences between the two experiments were the number of gramicidin channels and the use of K^+ in the single-channel experiment and the use

of Na^+ in the macroscopic conductance experiment. Figure 2 shows that the asymmetry of the instantaneous conductance is less than the asymmetry of the stationary conductance. (A more detailed description of the relaxation process is presented in the following paper (Fröhlich, 1979).) In Fig. 3*a*, the probability distribution of the height of the single-channel current jump at very low gramicidin concentrations is given for different potentials of both polarities. From this histogram a current-voltage curve was constructed (Fig. 3*b*). Close inspection of this curve and of the high frequency curve in Fig. 2 reveals that they have identical shapes.

As an aside, the rectification of the gramicidin single-channel conductance is consistent with the expected effect of the negative surface charge on the PS side of the bilayer. Since on the PS side there are more cations near the negatively charged membrane surface and therefore near the mouth of the gramicidin channel, the current of cations driven by the membrane potential is larger in the direction from the PS to the PE side than in the opposite direction. However, the asymmetry is not necessarily only due to the asymmetric charge distribution on the membrane surface, since we also observed asymmetric current-voltage curves in asymmetric bilayers composed of only neutral lipids (*see* below).

Since Figs. 2 and 3*b* showed that the shape of the single-channel conductance is the same as the shape of the instantaneous macroscopic conductance, the assessment of the rectification of the single-channel conductance can therefore be made with either type of current-voltage curve. In addition to these, we have also measured the instantaneous currents from voltage-step relaxation experiments. If the membrane potential is switched from a given value to a different voltage, the initial current before the onset of a relaxation (but after the capacitive current decayed which was faster than the time resolution of these experiments) is due to the conductance through the same number of channels as were open prior to the pulse. In contrast, the stationary conductance obtained after the relaxation is the product of the voltage-dependent number of channels and their individual conductances and therefore cannot give direct information on the single-channel conductance. The data shown in Fig. 4*b* were obtained with three different methods from the same membrane: by applying triangular voltage pulses of high frequency and from relaxation experiments involving single voltage steps and continuous voltage pulses (Fröhlich, 1979). They demonstrate that all three methods yield the same information about the single-channel conductance asymmetry. A convenient measure of the asymmetry of

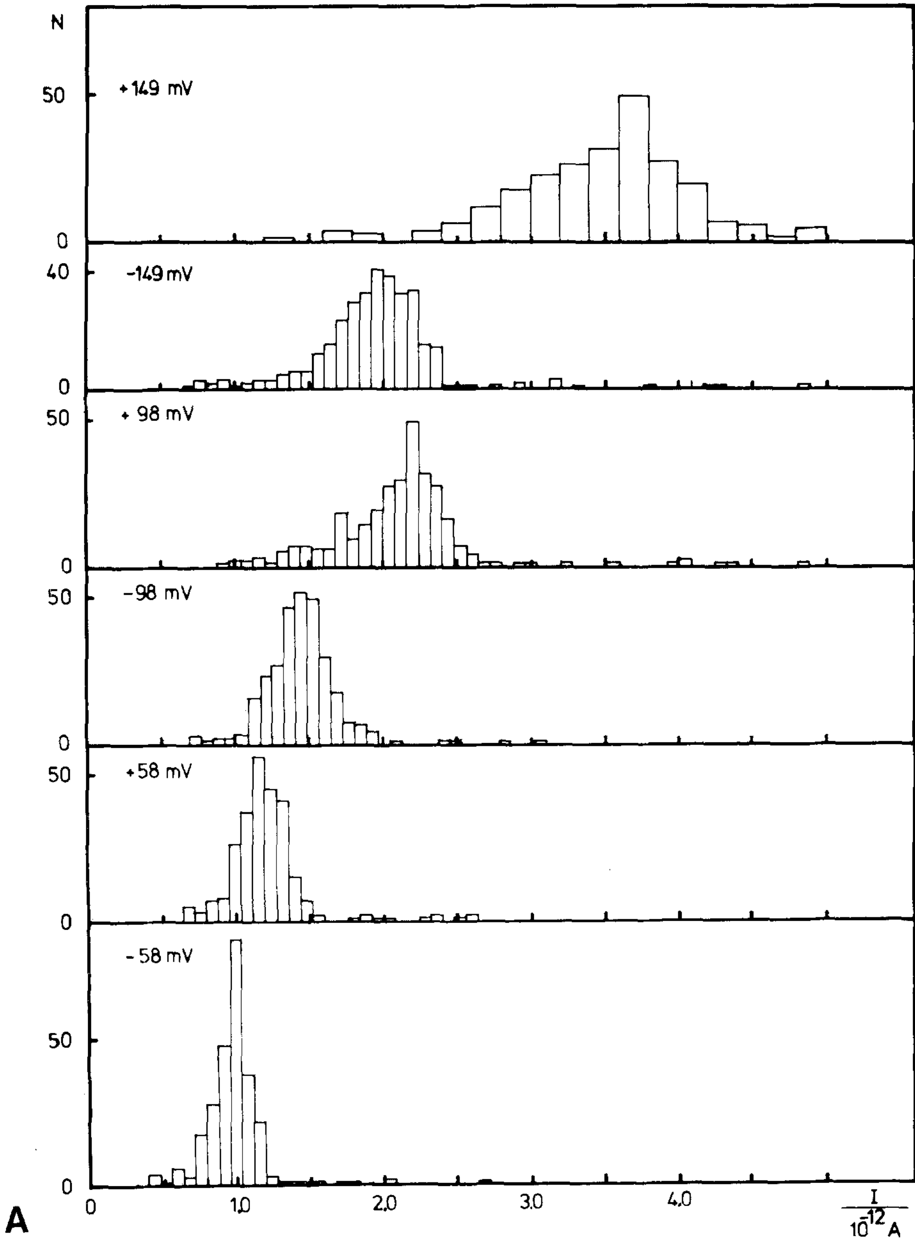


Fig. 3. (*A*): Histogram obtained from single-channel recordings at the indicated membrane potentials. Positive voltages mean positive on the PS side of the brain-PE/PS bilayer. Symmetric aqueous solution contained 100 mM KCl. *N* is the number of current jumps (single-channel events) of the magnitude indicated on the abscissa. (*B*): Current-voltage curve of single-channel conductance of a brain-PE/PS bilayer obtained by plotting the maxima of the current histograms in *A* as a function of the applied membrane potential V_m. Current of cations from PS to PE side is positive. Symmetrical aqueous solution contained 100 mM KCl

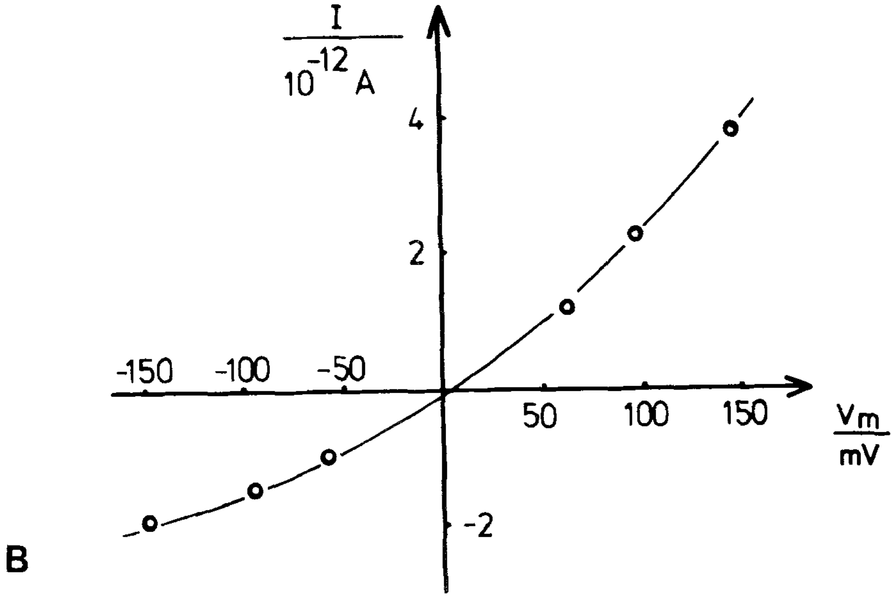


Fig. 3B

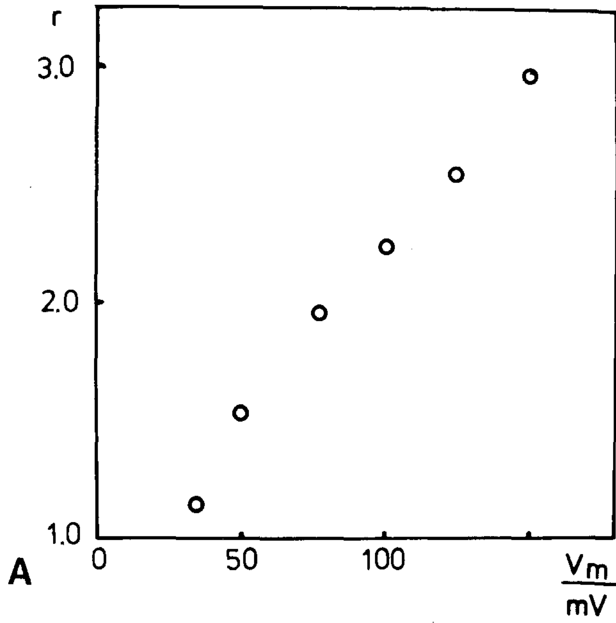
Table 1. Asymmetry of the gramicidin single-channel conductance in several asymmetric bilayers[a]

Membrane[b]	Rectification
Brain PE/PE 18:1	$r_{100}=1.4\pm0.2$ (2)
Brain PE/PC 18:1	$r_{50}=1.4$ (1)
	$r_{100}=1.7\pm0.2$ (4)
	$r_{150}=2.5$ (1)
Brain PE/PC 22:1	$r_{50}=1.7\pm0.2$ (14)
	$r_{100}=2.6\pm0.4$ (14)
	$r_{150}=3.3\pm0.6$ (12)
GME/brain PE	$r_{100}=1.6\pm0.4$ (11)
GMO/PC 22:1[c]	$r_{100}>2.5$

[a] r is the ratio of the currents at the voltage (in mV) indicated as subscript. 10 mM KCl was the bathing solution in all experiments. The number of membranes tested is given in parentheses.

[b] The lipid combination is arranged such that the current from the left lipid to the side of the right lipid is larger than in the opposite direction.

[c] The values varied over a very large range in different membranes so that only a lower limit is given.



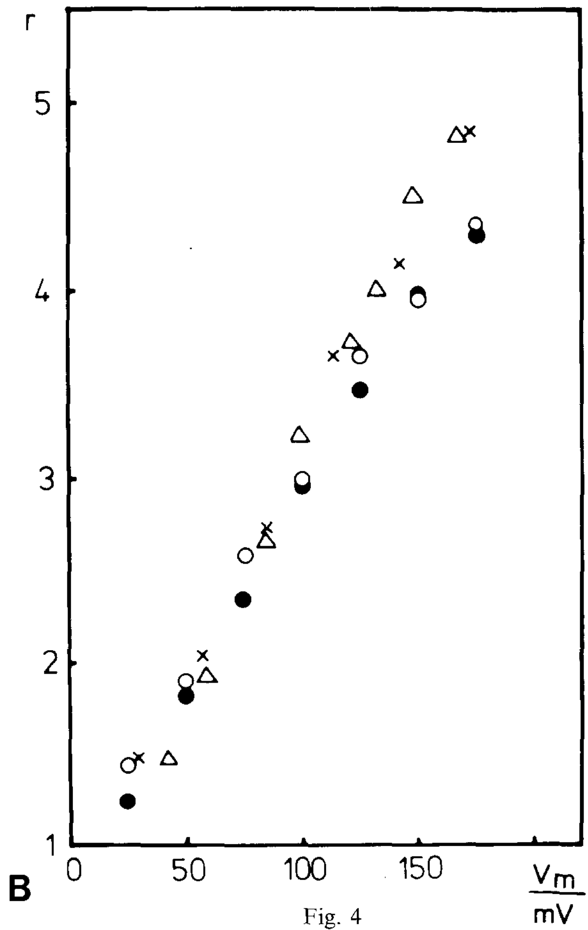


Fig. 4

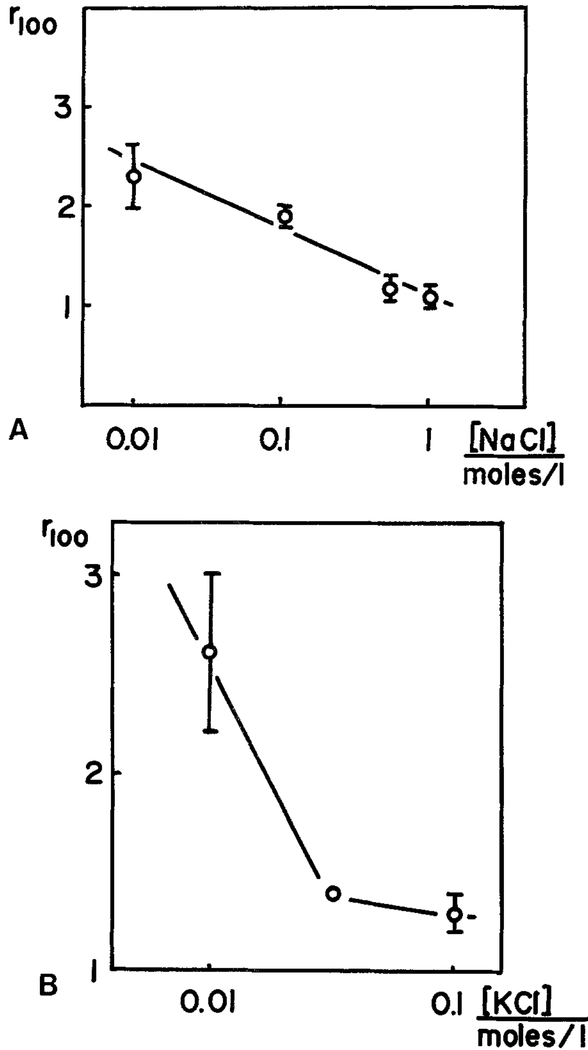


Fig. 5 (*A*): Dependence of current asymmetry of a brain-PE/PS bilayer on salt concentration in the aqueous phase. r_{100} is the ratio of the instantaneous currents at + and -100 mV. (*B*): Dependence of current asymmetry of a brain PE/PC 22:1 bilayer on salt concentration in the aqueous phase. r_{100} is the ratio of the instantaneous currents at + and -100 mV

Fig. 4. (*A*): Rectification *r*, measured as the ratio of currents at + and − voltages, of gramicidin channels as function of membrane potential. Data were obtained from a current-voltage curve at high frequency triangular voltage pulses in a brain-PE/PS bilayer. Symmetrical aqueous solution contained 10 mM KCl. (*B*): Rectification *r* of gramicidin pore as function of membrane potential in a brain-PE/PC 22:1 bilayer. Data were obtained from the same membrane by several methods indicated by the symbols. For details, *see* text. Symmetrical aqueous solution contained 10 mM KCl. ○, triangular pulse, 100 Hz; ●, triangular pulse, 10 Hz; ×, single voltage step experiment; △, continuous pulse experiment

the current-voltage curve is the ratio of the membrane currents (r) at equal but opposite membrane potentials. Figures 4*a* and *b* show r for a charged/neutral and a neutral/neutral asymmetric bilayer as a function of the membrane potential V_m. A roughly linear relationship between r and V_m is observed. Note that the asymmetry of the gramicidin pore in a neutral/neutral asymmetric bilayer can be stronger than the asymmetry in a bilayer with asymmetric charge distribution. Table 1 summarizes the r values at several potentials obtained in five different asymmetric bilayer systems.

The asymmetry of the single-channel conductance depends on the ion concentration in the aqueous phase. At higher concentrations the asymmetry decreases. Figure 5*a* and *b* shows that this is the case with both charged/neutral and neutral/neutral asymmetric bilayers.

Discussion

In this paper it was demonstrated that gramicidin induces a strongly asymmetric conductance in bilayers of asymmetric lipid distribution (Fig. 1). The asymmetry of the stationary conductance shown in Fig. 1 is believed to be due to two factors: the asymmetric single channel conductance and the asymmetric voltage dependence of the dimerization step which forms the channel. The asymmetry of the channel formation step is evident from an analysis of Fig. 2. Comparing the two branches of the *I-V* curve at high frequency sweeps, we can see that, upon decreasing the sweep frequency, the slope of the positive current branch increases and the slope of the negative current branch decreases. If the same experiment is performed with a symmetrical bilayer, both branches exhibit a steeper slope when the sweep frequency is lowered (not shown). This asymmetric voltage dependence of the dimerization step is described in more detail in the accompanying paper and is discussed further there (Fröhlich, 1979). The single-channel conductance behavior of the gramicidin pore is treated separately here from the dimerization reaction. Our justification lies in the fact that these processes can be experimentally separated in two ways: (i) kinetically (instantaneous *vs.* stationary conductance) and (ii) chemically: a covalently dimerized gramicidin analog has the same single-channel conductance as the hydrogen-bonded dimer in symmetrical bilayers, but has different on- and off-kinetics (Bamberg & Janko, 1977), and noise analysis of the ion current through

the covalent dimer exhibits the electrical behavior expected from a pore (Sauvé & Bamberg, 1978).

As previously pointed out, the asymmetry of the single channel conductance in neutral/charged asymmetric bilayers is compatible with the notion that it is influenced by the surface charge of the bilayer. A negatively charged bilayer possesses an electric double layer with a high concentration of the permeant cations near the surface (McLaughlin, 1977). As long as the bulk cation concentration far away from the membrane is lower than the concentration necessary to saturate the binding sites for the ions in the gramicidin molecule, the higher cation concentration near the mouth of the channel due to negative surface charges will lead to a higher flux through the pore in the direction away from the charged membrane side. This has been previously demonstrated by the use of a gramicidin analog carrying several negative charges near its mouth (Appell *et al.,* 1977). However, in view of the fact that the gramicidin pore is also asymmetric in asymmetric bilayers made only of neutral lipids, there is no value in trying to quantitatively correlate the observed asymmetric conductance with the asymmetry expected from a given surface charge density. With the available data, we cannot yet quantitatively separate the intrinsic asymmetry of the pore due to the asymmetry of the lipid environment from the asymmetry due to an asymmetric ion distribution. A similar conclusion has been reached in a recent study by Apell, Bamberg & Läuger (1979) who showed that neither the Gouy-Chapman theory nor the Stern treatment of surface charge effects on bilayers could quantitatively explain the difference between the single channel conductances in PS and PC bilayers.

It has been suggested (Haydon & Myers, 1973) that the dipole potential in a bilayer should affect the single-channel conductance in the same way as it affects the carrier-mediated conductance in bilayers (Hladky & Haydon, 1973; Andersen *et al.,* 1976; Latorre & Hall, 1976; McLaughlin, 1977). Szabo and McBride (1978) have presented evidence that this – though only to a small extent – could be the case. We do not think that the membrane dipole potential profile plays the only role in determining the single-channel conductance. Experiments with symmetric bilayers of dioleoyl-PC/decane, which were made asymmetric by adding phloretin to one side of the bilayer and thus changing the dipole potential on only one side at first, do not reveal any asymmetry in the single channel conductance, although the voltage-dependent dimerization is strongly asymmetric (*unpublished observation*). The possibility remains, however, that the phloretin, believed to change the dipole poten-

tial in a bilayer (Andersen *et al.*, 1976), has an additional effect on the gramicidin (e.g., by altering a membrane structural property) which fortuitously offsets the direct effect of the dipole potential. Since the dipole potential is considered to be independent of the ionic strength in the aqueous phase (Latorre & Hall, 1976), the results shown in Fig. 5 are additional evidence against the notion of a dipole effect.

Factors different from the electrostatic influence of membrane surface potential or dipole moment contribute to the potential profile along the transport pathway. It is well established now that the single channel conductance of gramicidin depends on the lipid which is used to form the bilayer (Bamberg *et al.*, 1976; Neher & Eibl, 1977; Kolb & Bamberg, 1977). The conductance can vary by a factor of two between lipids of different polar head groups (Bamberg *et al.*, 1976) even if the fatty acid side chains are identical (Neher & Eibl, 1977). In addition, the conductance is affected by the chain length of the fatty acid in lipids with the same polar head group (Kolb & Bamberg, 1977). Neher and Eibl (1977) established a correlation between the surface tension of the bilayers and the single-channel conductance and kinetics of the dimerization step in membranes made of PE and PC and intermediate homologs of different degrees of methylation in the head group, but with identical fatty acid compositon. A correlation, however, is not sufficient to prove a direct causal relationship between two parameters.

The single-channel conductance measurements in symmetrical membranes and the data presented here are consistent with the idea that the single-channel conductance is governed by the part of the pore monomer through which the ion enters the bilayer. The current from the PS to the PE side or monoglyceride to the PC side (Fig. 3 and Table 1) is larger than the current in the opposite direction, as predicted from the higher single-channel conductance in symmetrical bilayers of PS *vs.* PE and GMO *vs.* PC (Table 2)[3]. For the sake of clarity of the argument let us assume the simplest scheme possible to describe the basic characteristics of ion transport through the channel (which obviously does not

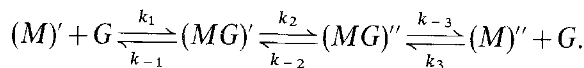
[3] There is no direct comparison available in the literature between PS and PE bilayers under identical conditions (fatty acid composition of lipids, salt composition, etc.). However, under the assumption that the concentration dependences of the conductance of gramicidin A and B in PE and PC bilayers are similar (because both lipids bear no net charge), one can use the data on PS and PC bilayers and on PC and PE bilayers shown in Table 2 to compare the conductances in PE and PS membranes. From this comparison it seems quite probable that at low cation concentrations (<100 mM) the single-channel conductance in PS bilayers is generally larger than in PE bilayers, despite the variation found among bilayers made of the same type of phospholipid but of different fatty acid composition and the somewhat different conductances of gramicidin A and B (Bamberg *et al.*, 1976).

Table 2. Comparison of reported values of the gramicidin single-channel conductances in symmetrical bilayers made from different phospholipids (the units are in nS/channel)

Electrolyte	PS	PC	PE	GMO	Authors
10 mM CsCl	42 (60)	2			Apell *et al.* (1979)[a]
100 mM CsCl	65 (65)	15			
1 M CsCl	66 (66)	46			
100 mM CsCl		9.5		15	Kolb & Bamberg (1977)[b]
1 M CsCl		26		76	
1 M CsCl		35	52	80	Bamberg *et al.* (1976)[c]
1 M KCl		18	16	35	
1 M KCl		41	69		Neher & Eibl (1977)[d]

[a] Diphytanol-PC and -PS/decane; 25 °C; 50–100 mV; the values in parentheses were obtained in the presence of EDTA; purified gramicidin A.
[b] GMO and dioleoyl-PC/decane; 25 °C; 100 mV; purified gramicidin A.
[c] GMO, dioleoyl-PC and -PE/decane; 25 °C; 100 mV; purified gramicidin B.
[d] Dipentadecylmethylidene-PC and -PE/decane; 25 °C; 100 mV.

explain all aspects of gramicidin conductance):



$$(M)' + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (MG)' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (MG)'' \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} (M)'' + G.$$

A cation M on one side of the membrane (′) complexes with a binding site in the mouth of the gramicidin channel facing the side (′). After translocation through the channel to the corresponding binding site on the other side (″) of the membrane, the cation is unloaded. In a symmetrical channel $k_1 = k_3$, $k_{-1} = k_{-3}$, and $k_2 = k_{-2}$. Within this framework the direction of the observed conductance asymmetry suggests that the gramicidin pore is asymmetric with respect to the rates at which the ions enter the channel onto the binding site (i.e., $k_1 \neq k_3$). It is not possible to tell if the exit rates k_{-1} and k_{-3} are asymmetric as well because at this time one can only draw conclusions about the rate-limiting step. Probably, the exit rate is not limiting in the overall rate of transport. If it were, one would expect the current from the GMO side (the entry side) to the PC side (the exit side) to be smaller than in the opposite direction, since the conductance in symmetrical PC bilayers is smaller than in symmetrical GMO bilayers (Table 2). The observed asymmetry is opposite to this prediction, indicating that the conductance is governed by the rate of transfer of the ion from the aqueous phase into the channel. The kinetic analysis of Levitt (1978) supports this notion. He found that the conductance is partially limited by the rate of going

from the bulk solution to a binding site in the channel near the interface. This is particularly valid in the case of low ion concentrations because the rate of loading the binding site from the aqueous phase becomes smaller with smaller ion concentrations. The possibility that an asymmetric lipid distribution may primarily affect the translocation step, thus making the rate of translocation from the binding site on one end of the channel to the other side different from the translocation in the opposite direction (i.e., $k_2 \neq k_{-2}$) is unlikely, although it cannot be excluded at this time. If the possibly asymmetric translocation step is rate determining and all other rate constants are symmetrical, one would expect the asymmetry of the conductance to be nearly the same at all ion concentrations. The fact that the asymmetry decreases with higher ion concentrations (Fig. 5*a* and *b*) gives support to the first explanation that the rate of entry into the channel is asymmetric in asymmetric bilayers. At low concentrations the (asymmetric) entry step is rate limiting in the overall conductance process but its rate can be expected to increase with increasing cation concentration. At high concentrations the (symmetric or at least less asymmetric) translocation step determines the conductance through the pore. Since the data of Hladky (1974) which Levitt used in his analysis (Levitt, 1978) are from membranes formed from only one lipid, a more detailed analysis of the asymmetry with Levitt's model awaits the corresponding measurements on membranes made of lipids used in this study.

At this state it is not possible to determine if only the entry step is affected by the lipid environment of the channel thus changing the affinity of the binding site for the cation, or if the exit step is altered in the same way leaving the affinity unchanged. Information on this question, however, can be obtained by studying the influence of the lipid on the concentration dependence of the conductance since the affinity of the site determines to a great deal the concentration at which the conductance saturates.

In a strict sense, the presented argument is only fully valid in the case of a channel occupied by only one ion at any time. There is increasing evidence, however, suggesting that the gramicidin channel is multiply occupied at high cation concentrations, and Urban, Hladky and Haydon (1978) have claimed that even at the relatively low salt concentrations of 10–100 mM there is a significant probability of a channel having two cations (with K^+ and Cs^+, less with Na^+). Since a multiple-site, multiple-occupancy model contains, depending on its precise nature, many more adjustable parameters than the very simple two-site, single-

occupancy model outlined above, it cannot be ruled out that under certain assumptions the above argumentation is not valid.

The mechanism by which the lipid surrounding the gramicidin pore alters the rate constants is unclear. The polar head groups play a role perhaps in the plane of the membrane-water interface where the dielectric properties are different with different lipids and determine the height of the energy barrier between the bulk aqueous phase and the binding site in the mouth of the channel. Alternatively, the conformation of the gramicidin channel may be susceptible to some structural properties of the lipid matrix such as fluidity characteristics or maybe even surface tension (Fröhlich, 1979; Neher & Eibl, 1977).

It is not unreasonable to suppose that a slight distortion of the helical backbone of the channel could lead to an altered potential energy profile seen by the ion inside the pore. The asymmetric voltage dependence of the gramicidin dimerization (Fröhlich, 1979) is evidence supporting this idea; a slight change of the polypeptide conformation depending on the lipid environment could be the cause of an altered interaction between the coupling ends of the two gramicidin monomers.

There is yet another explanation for the effect of ion concentration on the asymmetric conductance. So far it has been assumed the ions have a direct effect on the gramicidin molecules. We cannot exclude, however, that the ions influence the gramicidin behavior indirectly via the surrounding lipid. It has already been mentioned that the different lipids cause different single channel conductances, and there is some evidence in the literature that even monovalent cations can influence the melting characteristics of phospholipids (Simon *et al.*, 1975). Gramicidin may very well be a much more sensitive probe to such changes of the lipid structure than the methods previously employed.

In summary, the gramicidin single-channel conductance is asymmetric in bilayers of asymmetric lipid distribution. Since the rectification is also observed in membranes devoid of net surface charges, the asymmetry is an intrinsic channel property and not merely a consequence of an asymmetric ion distribution on the two sides of the membrane. The mechanism of how the single-channel conductance depends on the lipid in which the gramicidin is embedded remains unknown. It is possible, however, to draw qualitative conclusions from the concentration dependence of the asymmetry. In accordance with the kinetic analysis of Levitt (1978) of the symmetrical channel conductance, it can be concluded that the rate of complexation of an entering ion with a channel binding site at low ion concentrations is rate limiting and that the rate constant

of this process is influenced by the lipid surrounding the channel. At higher ion concentrations, the less asymmetric translocation rates appear to be rate determining.

The author thanks Drs. E. Bamberg, R. Benz and P. Läuger for many helpful discussions during this work and Dr. R.B. Gunn for his critique during the preparation of the manuscript. This work has been supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138) and U.S. Public Health Service grant HL 20365.

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