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# INFLUENCE OF MEMBRANE THICKNESS AND ION CONCENTRATION ON THE PROPERTIES OF THE GRAMICIDIN A CHANNEL

## AUTOCORRELATION, SPECTRAL POWER DENSITY, RELAXATION AND SINGLE-CHANNEL STUDIES

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## Summary

The properties of the gramicidin A channel in membranes made from a series of monoglycerides have been studied. In agreement with previous studies, the dissociation rate constant $k_D$ of the dimeric channel was found to increase strongly with increasing chain length of the monoglyceride, corresponding to a decrease of the mean life-time of the channel. The value of $k_D$, however, was not strictly correlated with the membrane thickness, as seen from a comparison of membranes with different solvent content. Furthermore, the life-time of the channel increased with the concentration of the permeable ion. This effect was tentatively explained by an electrostatic stabilization of the channel.

The single-channel conductance $\Lambda$ was found to decrease with increasing membrane thickness $d$, if $d$ was varied by increasing the chain length of the lipid. On the other hand, if $d$ was changed by varying the solvent content of the membranes formed from one and the same lipid, $\Lambda$ remained constant. These observations were explained by the assumption of local inhomogeneities in the membrane thickness. A striking difference between the $\Lambda$ values obtained from autocorrelation analysis in the presence of many channels ($\Lambda_a$) and those obtained from single-channel experiments ($\Lambda_{sc}$) occurred with membranes from longer chain-length monoglycerides. This difference disappeared at low ion concentrations. Electrostatic interactions between channels in local clusters were proposed for an interpretation of these findings.

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## Introduction

The cation permeability of lipid bilayers is increased in the presence of the linear pentadecapeptide gramicidin A (for a survey of the literature see refs. 1 and 2). There is evidence that gramicidin A creates dimeric channels consisting of two monomeres that are linked by hydrogen bonds [3—9]. Information about the kinetics of the dimerization reaction has been obtained by voltage-jump relaxation experiments [5,8,10]. Recently, an alternative method, correlation analysis, has been used to study the kinetics of gramicidin channel formation in lipid bilayer membranes [6,11]. This method has the advantage that the measurements are carried out while the system is in an equilibrium or stationary state. In addition to the kinetic parameters of channel formation, correlation analysis yields also information on the unit conductance step associated with the opening and closing of a channel. The results of the correlation analysis for gramicidin A-doped lipid bilayer membranes made from dioleoyl phosphatidylcholine membranes agreed closely with the values of relaxation and single-channel experiments [6,11]. An important question concerns the dependence of the single-channel parameters of gramicidin A on membrane thickness [3] and ionic strength. We have studied these problems by measuring the single-channel conductance as well as the rate constants of dissociation and association of gramicidin A in membranes made from a series of monoglycerides with increasing chain length of the *cis*-mono-unsaturated fatty acid. The membranes were either of the Mueller-Rudin [12] or Montal-Mueller type [13]. Assuming a channel length of about 25—30 Å [4] the ratio of channel length to membrane thickness was varied between about 0.4 and 1.1. It was also examined if there is an influence of ionic strength on the dimerization reaction. We found that increasing the ionic strength increases the stability of the channel.

Furthermore, we show that the values of channel conductance obtained from single-channel experiments and from noise analysis in the presence of many channels may differ significantly under certain circumstances.

## Materials and Methods

Monoglycerides with $C_{16}$ to $C_{24}$ *cis*-mono-unsaturated fatty acid chains monopalmitolein, monoolein, monoeicosenoin, monoerucin, monoervonin) were obtained from Nu Chek Prep, Elysian, Minn., U.S.A. All the samples consisted mainly of the α-isomer with small amounts of the β-isomer; they were used without further purification. 1,2-Dioleoyl-sn-glycerol-3 phosphorylcholine (dioleoyl phosphatidylcholine) was synthesized and chromatographically purified in our laboratory by K. Janko. Purified gramicidin A was a gift from Dr. E. Gross, Bethesda, Md. Gramicidin was added from a methanolic stock-solution to the electrolyte solution. Black lipid membranes were formed in the usual way from a 1—2% (w/v) lipid solution in *n*-decane or *n*-hexadecane [14] in a thermostated Teflon cell filled with an aqueous electrolyte solution at 25°C. Since the monoglycerides with longer fatty acid chains ($C_{20}$ to $C_{24}$) are little soluble in *n*-alkanes, the mixture was briefly heated to 40°C to obtain a clear solution [15]. The membrane area was between $3 \cdot 10^{-2}$ and $8 \cdot 10^{-3}$ $cm^2$ in the case of the autocorrelation and relaxation experiments and $4 \cdot 10^{-3}$ $cm^2$ for the measurement of single-channel conductances. Bilayer membranes from monolayers were formed according to the method of Montal and Mueller [13,15]. A thermostated Teflon cell was separated into two halves by a 12.5 μm thin Teflon septum. The diameter of the hole in the septum on which the membrane was formed was about 0.2 to 0.3 mm. Membrane formation was facilitated by coating the surroundings of the hole with a thin layer of vaseline [15].

Electrical relaxation experiments were carried out as described earlier [5] by applying a fast voltage step to the membrane through silver-silver chloride electrodes. The measurements of the current fluctuations arising from the formation and disappearance of single channels were carried out as described in a previous paper [8].

Autocorrelation analysis of the current noise was carried out with a Honeywell-Saicor-43A correlator, as described previously [6]. The membrane current was preamplified (Analog Devices Model 52 K) and passed through an eight-pole Butterworth filter (Krohn-Hite Model 3342). The high and low pass filter position was set about 10 times above and below $f_c = 1/2\pi\tau_c$ where $\tau_c$ denotes the correlation time of the channel-noise. An exception was the experiment with monopalmitolein/*n*-hexadecane membranes where the ratio of $f_c$ to the high pass cut-off frequency ($8 \cdot 10^{-3}$ Hz) was only about 4. In all cases studied here the influence of filter characteristics on the shape and amplitude of the autocorrelation function was negligible (Kolb, H.-A. and Boheim, G., in preparation). The band-limited signal was amplified by a dc-coupled amplifier (Princeton Applied Research Model 113) and analyzed by the correlator. The RC-time of the feedback circuit of the preamplifier was set to about $10^{-4}$ s throughout. The reproducibility of the autocorrelation function $\tilde{C}(\tau)$ depends on the stability of the mean membrane current during the time of the analysis. An increase or decrease of the mean current during the sampling time of the correlator produces a positive or negative drift of the function $\tilde{C}(\tau)$ calculated by the correlator. In most cases the offset due to this drift was less than 15% of the amplitude of the autocorrelation function; measurements with larger drift were discarded. In order to reach quasi steady-state conditions, all measurements were carried out 15 to 30 min after membrane formation. The output of the correlator $\tilde{C}(\tau)$ could be described by a single exponential $C(\tau)$ superimposed by a constant offset $C_\infty$:

$$\tilde{C}(\tau) = C(\tau) + C_\infty = C_0 \cdot \exp(-\tau/\tau_c) + C_\infty$$

where $C_0 = C(0)$ is equal to the variance of the current and $\tau_c$ is the correlation time (see below). The three constants $C_0$, $C_\infty$ and $\tau_c$ were determined by fitting the data to an exponential by the method of least squares. The resolution of the Saicor-43A for different signal-to-noise ratios was checked with a noise generator (Wavetek Model 132). A sine function of varying frequency (10 Hz to 1 kHz) was used as a signal waveform. A voltage signal-to-noise ratio of about 0.25 could be resolved for a noise bandwidth up to 1 kHz. Furthermore, experiments were carried out in which the membrane current noise was simultaneously processed by the correlator and a real time spectrum analyzer (Honeywell-Saicor-52B). For the case of spectral analysis, the current noise was amplified in the same way as above. The feedback resistor of the preamplifier was set to $5 \cdot 10^7\,\Omega$. A feedback capacitor of 2 pF was required for an unattenuated amplifier response in a frequency range up to 50 Hz. The spectrum analyzer used allows the average of a selected number of spectra of the power density $S(f)$ to be displayed as logarithmic presentation vs. frequency $f$.

The frequency output of the instrument was fed into a suitable adapted log-amplifier (Analog Devices Model 755N) so that a power density spectrum log $S(f)$ vs. log $f$ could be obtained. The output of the spectrum analyzer was calibrated by recording the power levels of different source resistors (1—6 MΩ). The corresponding thermal noise computed from the Nyquist relation agreed closely with the measured white-noise power level.

## Results

The rate constants of formation and dissociation of the dimeric channel were obtained from autocorrelation and voltage-jump relaxation experiments. After a sudden displacement of the voltage across the membrane, the dimer concentration of gramicidin A in the membrane shifts to a new stationary value. The relaxation time $\tau_c$ of the dimerization reaction of gramicidin

$$G + G \underset{k_D}{\overset{k_R}{\rightleftharpoons}} G_2$$

is given by [5]

$$\frac{1}{\tau_c} = k_D + 4\sqrt{\frac{k_R \cdot k_D \cdot \lambda}{L \cdot \Lambda}} \qquad (1)$$

$k_R$ and $k_D$ are the rate constants of association and dissociation respectively, $L$ is Avogadro's number, $\lambda$ the mean steady-state conductance of the membrane and $\Lambda$ the single-channel conductance of gramicidin A. Fig. 1 shows the results of relaxation experiments with monoolein/*n*-hexadecane membranes in the presence of 1 M NaCl. According to Eqn. 1 the dissociation constant $k_D$ is given by the intercept of the straight line with the $1/\tau_c$ axis.

The autocorrelation method may be used besides the voltage-jump technique to obtain the relaxation time $\tau_c$ under steady-state voltage-clamp conditions. The instant membrane current $J(t)$ exhibits statistical fluctuations arising from the random formation and dissociation of dimers:

$$J(t) = \bar{J} + \delta J(t)$$

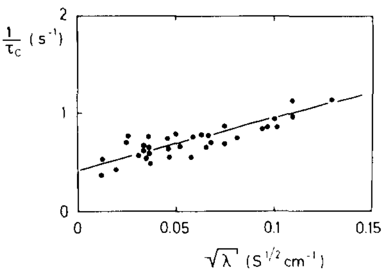

Fig. 1. Reciprocal of the relaxation time $\tau_c$ as a function of the square root of the mean membrane conductance $\lambda$ for a voltage-jump of $V_m$ = 100 mV. Glycerylmonoolein/*n*-hexadecane membranes in 1 M NaCl. The straight line was drawn according to linear regression.

$\bar{J}$ denotes the average current and $\delta J(t)$ the fluctuating part of $J(t)$. Using the fluctuation dissipation theorem [16], the autocorrelation function of the current $J(t)$ can be written as [6]:

$$C(\tau) = \overline{(\delta J)^2} \cdot \exp(-\tau/\tau_c) \tag{2}$$

where $\tau_c$ is the macroscopic relaxation time given by Eqn. 1 and $\overline{(\delta J)^2}$ the mean square of the current fluctuation or the variance of $J(t)$. The single-channel conductance $\Lambda$ can be determined from the variance of the membrane current according to the relation [6]:

$$\frac{\overline{(\delta J)^2}}{\bar{J}^2} = \frac{\Lambda}{\lambda \cdot A} \cdot \frac{1}{1 + 4\sqrt{K \cdot \lambda / L \cdot \Lambda}} \tag{3}$$

where $A$ denotes the membrane area and $K = k_R/k_D$ the equilibrium constant of the dimerization reaction. In the limit of low $\lambda$, the square root in the denominator of Eqn. 3 may be neglected:

$$\frac{\overline{(\delta J)^2}}{\bar{J}^2} \approx \frac{\Lambda}{A \cdot \lambda} \tag{4}$$

Therefore, only small values of $\lambda$ ($\lambda \leqslant 10^{-4}$ S $\cdot$ cm$^{-2}$) were used to determine $\Lambda$ from Eqn. 4. The autocorrelation function measured for monoglycerides with $C_{16}$ to $C_{24}$ *cis*-mono-unsaturated fatty acid chains at different gramicidin and ion concentrations could in each case be well described by a single exponential (Eqn. 2). The plot of $1/\tau_c$ as function of $\sqrt{\lambda}$ gives a straight line within the experimental error.

Figs. 2a and b represent two examples for monoolein/*n*-hexadecane and monoeicosenoin/*n*-hexadecane membranes. Fig. 2a shows a significant variation of the kinetic parameters with ionic concentration between 0.1 M and 1 M CsCl. $k_D$ increases with decreasing ion concentration. The values of $k_D$ obtained for different systems are listed in Table I. It is seen that at a constant ion concentration $k_D$ increases with increasing chain length of the fatty acid by about a factor of 400 between $C_{16}$ and $C_{24}$ (see also Fig. 3). $k_D$ seems to be independent of the nature of the ion as the comparison between Fig. 1 and the lower curve of Fig. 2a shows. Relaxation and autocorrelation experiments were carried out at a different voltage, 100 mV and 30 mV; it is known from other studies, however, that the voltage dependence of $k_R$ and $k_D$ is rather small in this voltage range. The dimerization model requires that $k_D$ should be equal to the reciprocal value of the mean channel life-time $\tau^*$ determined from single-channel experiments. As Table I shows, the values of $k_D$ and $1/\tau^*$ determined by the different methods are in close agreement. For the determination of the association constant $k_R$ from the slope of $1/\tau_c$ vs. $\sqrt{\lambda}$ (Eqn. 1), the single-channel conductance $\Lambda$ has to be known. According to Eqn. 4 a plot of $\overline{(\delta J)^2}/\bar{J}^2$ vs. $1/\lambda \cdot A$ in the limit of low $\lambda \cdot A$ yields an estimate of $\Lambda$. For monoolein/*n*-hexadecane membranes in 1 M CsCl one obtains $\Lambda$ = 82 pS (Fig. 4). Using this value and the slope of the lower curve in Fig. 2a, Eqn. 1 yields an association constant of $k_R = 2.2 \cdot 10^{13}$ cm$^2$ $\cdot$ mol$^{-1}$ $\cdot$ s$^{-1}$ for an applied voltage of 30 mV. Dilution to

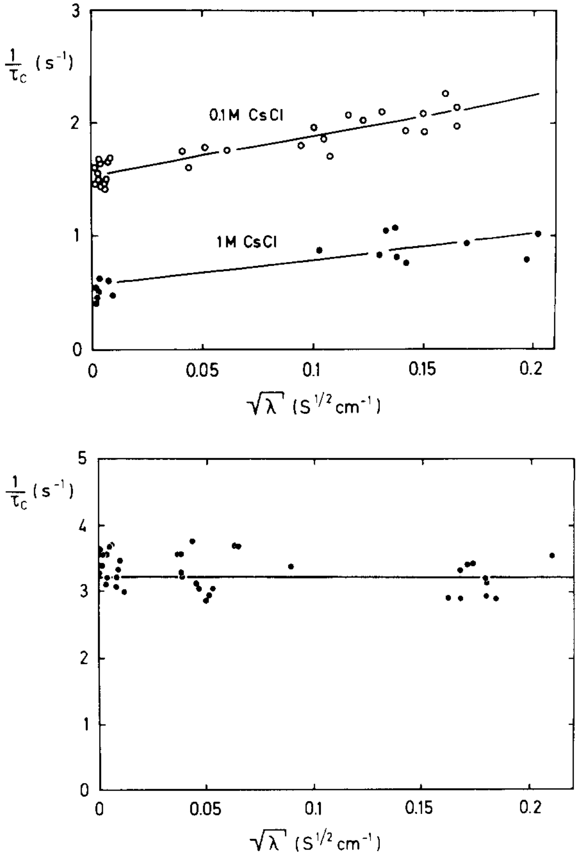

Fig. 2. (a) Reciprocal of the correlation time $\tau_c$ vs. $\sqrt{\lambda}$ for an external voltage $V_m$ = 30 mV. Glycerylmonoolein/*n*-hexadecane membranes in the presence of 1 M CsCl, (●) or 0.1 M CsCl, (○). The straight lines were drawn according to linear regression. (b) $1/\tau_c$ vs. $\sqrt{\lambda}$ for an external voltage $V_m$ = 30 mV. Glycerylmonoeicosenoin/*n*-hexadecane membranes in 1 M CsCl. The straight line was drawn according to linear regression setting the slope of the regression line to zero. Each point has been obtained from a different membrane.

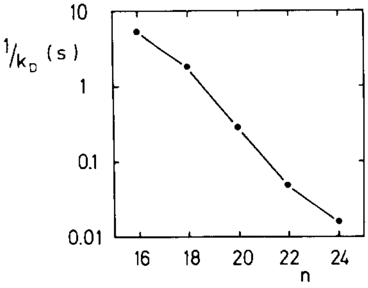

Fig. 3. Mean life-time $1/k_D$ of the gramicidin A channel in membranes of different monoglycerides. *n* is the number of carbon atoms of the *cis*-mono-unsaturated fatty acid of the monoglyceride. The solvent was *n*-hexadecane. The $k_D$ values were measured in 1 M CsCl by the autocorrelation method (Table I), except for membranes of monopalmitolein where 1 M NaCl was used.

TABLE I

RESULTS OF AUTOCORRELATION, RELAXATION AND SINGLE-CHANNEL EXPERIMENTS WITH DOPED LIPID BILAYER MEMBRANES IN THE PRESENCE OF GRAMICIDIN A AT 25°C

$k_R$ and $k_D$ are the rate constants of association and dissociation obtained from autocorrelation experiments; values of $k_R$ and $k_D$ from relaxation experiments are given in brackets [10]. $\Lambda_a$ and $\Lambda_{sc}$ are the values of the channel conductance obtained from autocorrelation and single-channel experiments, respectively. $\tau^*$ is the mean life-time of the channel (from single-channel studies). The autocorrelation analysis was done at $V_m$ = 30 mV, single-channel and relaxation experiments at $V_m$ = 100 mV. The values for the hydrocarbon thickness $d$ of the bilayer membranes were taken from ref. 15.

| Solvent | $d$ (nm) | Cation | $1/\tau^*$ ($s^{-1}$) | $k_D$ ($s^{-1}$) | $k_R$ ($10^{12}$ $cm^2 \cdot mol^{-1} \cdot s^{-1}$) | $\Lambda_a$ (pS) | $\Lambda_{sc}$ (pS) |
|---|---|---|---|---|---|---|---|
| monopalmitolein (16 : 1) | | | | | | | |
| $n$-Decane | 4.18 | 1 M $Na^+$ | — | 1.89 | 180 | 28 | — |
| $n$-Hexadecane | 2.18 | 1 M $Na^+$ | — | 0.19 | — | 26 | — |
| monoolein (18 : 1) | | | | | | | |
| $n$-Decane | 4.77 | 1 M $Na^+$ | 4.76 | 3.9 | — | 26 | 25 |
| $n$-Decane | 4.77 | 1 M $Cs^+$ | 4.17 | 3.7 | — | 82 | 90 |
| $n$-Hexadecane | 3.18 | 1 M $Na^+$ | 0.54 | 0.5 (0.45) | (50) | 28 | 24 * |
| $n$-Hexadecane | 3.18 | 1 M $Cs^+$ | — | 0.45 | 22 | 76 | 84 |
| $n$-Hexadecane | 3.18 | 0.1 M $Cs^+$ | — | 1.5 | 4.7 | 15 | 17.5 |
| monoeicosenoin (20 : 1) | | | | | | | |
| $n$-Hexadecane | 3.73 | 1 M $Na^+$ | — | 2.9 | — | 23 | — |
| $n$-Hexadecane | 3.73 | 1 M $Cs^+$ | — | 3.3 | $\lesssim 3$ | 51 | — |
| monoerucin (22 : 1) | | | | | | | |
| $n$-Decane | 5.84 | 1 M $Na^+$ | — | 10.2 | — | 5.8 | — |
| $n$-Hexadecane | 4.85 | 1 M $Na^+$ | — | 18.4 | — | 8.0 | — |
| $n$-Hexadecane | 4.85 | 1 M $Cs^+$ | 23.3 | 19.2 | — | 23 | 65 |
| $n$-Hexadecane | 4.85 | 0.1 M $Cs^+$ | — | 31.3 | — | 10 | 13 |
| Solvent-free | 3.15 | 1 M $Na^+$ | 1.8 | 2.8 | — | 7.8 | 20 |
| Solvent-free | 3.15 | 1 M $Cs^+$ | 1.39 | (2.02) | — | — | 62 |
| mononervonin (24 : 1) | | | | | | | |
| $n$-Hexadecane | 6.86 | 1 M $Cs^+$ | — | 66.7 | — | 1.1 | — |
| $n$-Decane | 6.76 | 1 M $Cs^+$ | ≈100 | — | — | — | 7.9 |

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* This value is in close agreement with that of Hladky and Hayden [3].

0.1 M CsCl induces a five-fold decrease of $\Lambda$ determined by Eqn. 4 (see Fig. 4) and a significant decrease of $k_R$ to $4.7 \cdot 10^{12}$ $cm^2 \cdot mol^{-1} \cdot s^{-1}$. The theoretical curve in Fig. 4, which was drawn according to Eqn. 3, agrees closely with the experimental values in the range $\lambda \cdot A \leqslant 10^{-4}$ S, but with increasing $\lambda \cdot A$ the experimental values decrease faster than predicted by Eqn. 3. This deviation was observed for all the systems investigated, independent of ion concentration and membrane lipid composition. For comparison, $(\delta J)^2/J^2$ vs. $1/\lambda \cdot A$ was plotted for a membrane with increased hydrocarbon thickness as the monoeicosenoin/$n$-hexadecane membrane (Fig. 5). The theoretical curve was drawn using Eqn. 3 with $\Lambda$ = 51 pS and $4\sqrt{K \cdot \lambda/L \cdot \Lambda} << 1$. (According to Table I the equilibrium constant $K = k_R/k_D$ is less than $9.1 \cdot 10^{11}$ $cm^2 \cdot mol^{-1}$.) Table I gives a survey of the results for $k_R$, $k_D$ and $\Lambda$ obtained for different monoglyceride membranes and different ionic solutions. In 1 M CsCl, the value of $\Lambda$ obtained by single-channel experiments ($\Lambda_{sc}$) decreases with increasing thickness of the membrane (monoolein/$n$-hexadecane to monervonin/$n$-decane) by

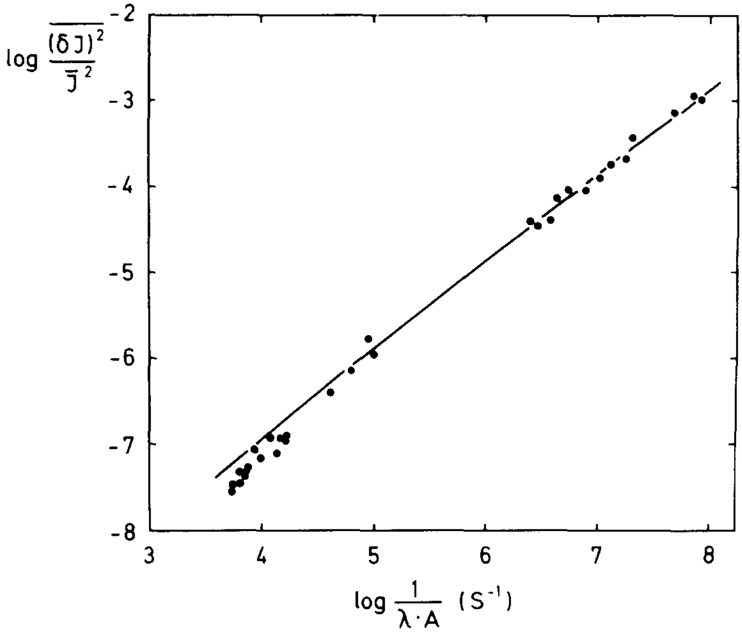


Fig. 4. $\overline{(\delta J)^2}/\bar{J}^2$ vs. the reciprocal of the mean membrane conductance $\lambda \cdot A$ for glycerylmonoolein/$n$-hexadecane membranes in 0.1 M CsCl. ($V_m$ = 30 mV). The theoretical curve has been drawn according to Eqn. 3 with $\Lambda_a$ = 76 pS. For the equilibrium constant $K = k_R/k_D$ a value of $4.1 \cdot 10^{13}$ cm$^2 \cdot$ mol$^{-1}$ was used.

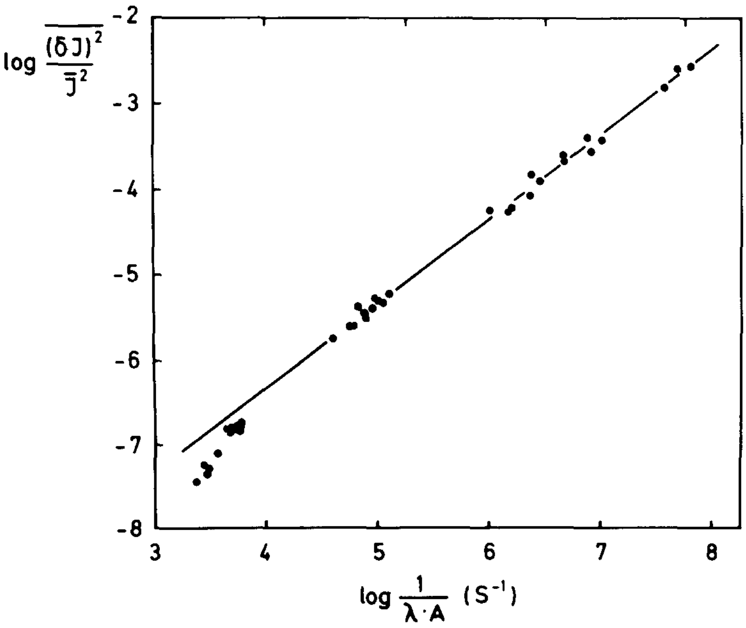


Fig. 5 $\overline{(\delta J)^2}/\bar{J}^2$ vs. $1/\lambda \cdot A$ for glycerylmonoerucin/$n$-hexadecane membranes in 1 M CsCl ($V_m$ = 30 mV). The theoretical curve has been drawn according to Eqn. 3 with $\Lambda_a$ = 51 pS. As the equilibrium constant $K = k_R/k_D$ is less than $9.1 \cdot 10^{11}$ cm$^2 \cdot$ mol$^{-1}$ (Table I), the term containing $K$ in Eqn. 3 was neglected.

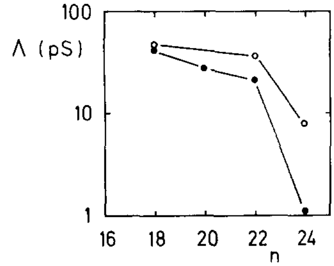
a factor of about 11. Preliminary data were presented in ref. 10. On the other hand, for the same variation of hydrocarbon thickness the value of $\Lambda$ determined by autocorrelation analysis ($\Lambda_a$) decreases by a factor of about 70 (see also Fig. 6). The discrepancy between $\Lambda_a$ and $\Lambda_{sc}$ seems to be mainly dependent on the chain length of the fatty acid and less on the solvent, since we find this to the same extend for solvent containing as for solvent free monoerucin membranes. Interestingly, dilution to 0.1 M CsCl removes almost completely the discrepancy between the $\Lambda$ values obtained by autocorrelation and single-channel analysis.

Measurements of the spectral power density of the current noise were performed in addition to the autocorrelation analysis. Both quantities, the autocorrelation function and the spectral power density are correlated by the Wiener-Khintchie transformation [17,18] and therefore yield essentially the same information. In this way, the value of $\Lambda$ may be obtained by a second, independent experimental method under conditions where many channels are present in the membrane. Spectral power density measurements may therefore be used as an additional check that the difference between $\Lambda_a$ and $\Lambda_{sc}$ is real and not caused by instrumental artefacts. The upper trace in Fig. 7 shows the spectral power density of the membrane current noise for a typical experiment with a monoerucin/*n*-hexadecan membrane in the presence of 1 M CsCl. The theoretical curve was drawn according to the relation:

$$S_J(f) = \frac{4 \cdot \tau_c \cdot \overline{(\delta J)^2}}{1 + (2\pi\tau_c f)^2} \tag{5}$$

which may be calculated from the Wiener-Khintchie transformation of the autocorrelation function (Eqn. 1). The corner frequency $f_c = 1/2\pi\tau_c$ and the variance $\overline{(\delta J)^2}$ were determined by the simultaneously processed autocorrelation function. As it can be seen from Fig. 7, there is a reasonable agreement between the spectral power density and the theoretical curve given by Eqn. 5. The value of $(\delta J)^2$ determined by autocorrelation and spectral analysis agreed within 10—20% for different membranes of the system described above. Therefore, a methodical bias in the determination of $\overline{(\delta J)^2}$ by autocorrelation analysis can be excluded. The lower trace in Fig. 7 shows a control experiment in which the current noise was recorded from a gramicidin-free membrane with an external resistor of 5.4 MΩ simulating the gramicidin-induced membrane conductance of the experiment above. The conductance of the bare membrane was less than $2.5 \cdot 10^{-8}$ S $\cdot$ cm$^{-2}$. The corresponding thermal noise level of the 5.4 MΩ resistor was computed from the Nyquist relation and is given by the dashed line. The thermal power density is four orders of magnitude below the spectral power density of a gramicidin A-doped membrane of the same resistance. Occasionally, monoerucin/*n*-hexadecane membranes were obtained which had an increased membrane conductance in the absence of gramicidin (more than ten times of the normal value). In these cases the spectral power density showed a $1/f^{\alpha}$ ($\alpha \simeq 1.1$) behaviour.

Table II presents the correlation time $\tau_c$ and the single-channel conductance $\Lambda_a$determined by autocorrelation analysis for a concentration range between 1 M and $10^{-3}$ M CsCl. The gramicidin concentration was chosen in such a way



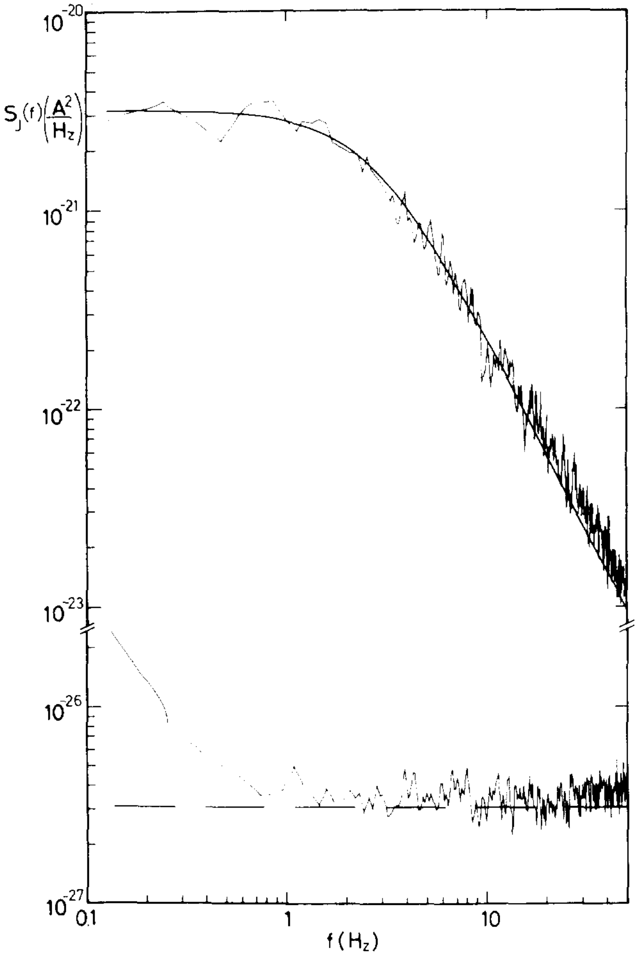


Fig. 6. Channel conductance Λ as obtained by autocorrelation analysis (●, $\Lambda_a$) and single-channel studies (○, $\Lambda_{sc}$). *n* is the number of carbon atoms in the *cis*-mono-unsaturated fatty acid of the monoglyceride. The solvent was *n*-hexadecane, the aqueous phase contained 1 M CsCl (values were taken from Table I).

Fig. 7. Spectral power density of the current noise $S_J(f)$ vs. frequency $f$ for glycerylmonoerucin/*n*-hexadecane membranes in 1 M CsCl at $V_m$ = 30 mV and 25°C. The upper trace represents the spectral power density for gramicidin A doped membrane with a mean macroscopic conductance of $2.4 \cdot 10^{-5}$ S · cm$^{-2}$. The theoretical curve was drawn according to Eqn. 5 using the values $\overline{(\delta J)^2} = 1.4 \cdot 10^{-20}$ A$^2$ and $\tau_c$ = 59 ms which were determined by the simultaneously processed autocorrelation function. The lower trace shows a control experiment as described in the text. The dashed line represents the level of the corresponding thermal noise of a 5.4 MΩ resistor calculated by the Nyquist relation. The spectral power densities shown are an average of 16 spectra, respectively.

that the macroscopic conductance was less than $10^{-4}$ ($10^{-5}$) S · cm$^{-2}$ for 1 M (0.1 M) CsCl, respectively, and between $5 \cdot 10^{-7}$ and $10^{-5}$ S · cm$^{-2}$ for lower ionic concentrations. In this range of conductances $\tau_c$ coincides virtually with $1/k_D$. Independently of the membrane forming lipid, monoolein, monoerucin and dioleoyllecithin, a reduction of the ionic strength induces a decrease of $\tau_c$ by a factor of 2—3 which appears to approach an asymptotic value for ion concentrations below $10^{-2}$ M. $\Lambda_a$ was calculated from the variance of the membrane current using Eqn. 4. As can be seen from Table II, $\Lambda_a$ changes almost linearly in the concentration range of $10^{-3}$ M to $10^{-1}$ M.

TABLE II

CORRELATION TIME $\tau_c$ AND SINGLE-CHANNEL CONDUCTANCE $\Lambda_a$, AS DETERMINED BY AUTOCORRELATION ANALYSIS, AS FUNCTION OF ION CONCENTRATION

$V_m = 30$ mV, $T = 25^\circ$C.

| Membrane composition | Channel parameters | Ion concentration (M) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CsCl | | | | NaCl | | LiCl | | |
| | | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-2}$ | $5 \cdot 10^{-3}$ | $10^{-2}$ | $5 \cdot 10^{-3}$ | $2.5 \cdot 10^{-3}$ |
| Monoolein/ | $\tau_c$(s) | 1.7 | 0.67 | 0.45 | 0.42 | | | | | |
| *n*-Hexadecane | $\Lambda_a$(pS) | 76 | 15 | 2.6 | 0.25 | | | | | |
| Monoerucin/ | $\tau_c$(s) | 0.048 | 0.034 | 0.024 | 0.022 | 0.026 | 0.024 | 0.023 | 0.022 | 0.023 |
| *n*-Hexadecane | $\Lambda_a$(pS) | 23 | 10 | 1.5 | 0.2 | 0.4 | 0.24 | 0.2 | 0.098 | 0.087 |
| Dioleoyl phosphatidylcholine | $\tau_c$(s) | 0.53 | 0.25 | 0.15 | 0.11 | | | | | |
| *n*-Decane | $\Lambda_a$(pS) | 26 | 9.5 | 1.5 | 0.4 | | | | | |

TABLE III

ION SELECTIVITY OF THE GRAMICIDIN A CHANNEL IN MEMBRANES OF DIFFERENT COMPOSITION

The ratios $\Lambda_i/\Lambda_{Na}$ of the single-channel conductances as determined by autocorrelation analysis are given. The different cations were present as chlorides at a concentration of 1 M. (i = Li, Cs).

| Membrane composition | Cation | | |
|---|---|---|---|
| | $Li^+$ | $Na^+$ | $Cs^+$ |
| Monoolein/*n*-hexadecane | 0.23 | 1.0 | 2.5 |
| Monoerucin/*n*-hexadecane | 0.18 | 1.0 | 2.9 |
| Dioleoyl phosphatidylcholine/*n*-decane | — | 1.0 | 2.9 |

In a series of experiments the limit of detection of $\Lambda_a$ by autocorrelation analysis was evaluated for membrane systems which showed a correlation time $\tau_c$ in the 20 ms range in the presence of gramicidin A. For this case, $\Lambda_a$ was determined for monoerucin/*n*-hexadecane membranes at constant gramicidin concentration but decreasing ion concentration. For concentrations of CsCl less than $10^{-3}$ M, diffusion polarization at the electrodes could not be excluded. Therefore, LiCl was choosen as permeating ion since a definite value of $\Lambda_a$ is reached at about a 10-fold higher concentration of lithium in the aqueous phase compared with caesium. As can be seen from Table II, a value of $\Lambda_a$ = $8.7 \cdot 10^{-14}$ S could be detected in the presence of 2.5 mM LiCl for the set up used in this study.

The determination of $\Lambda_a$ is limited by the signal-to-noise ratio of the experimental set up. If one takes into account the thermal noise of the membrane and the current-voltage noise specifications of the preamplifier as background noise, the signal-to-noise ratio may be estimated according to Eqn. A1 of the paper of Poussart ref. 19. An unfavourable system with respect to the signal-to-noise ratio of a gramicidin A doped monoerucin/*n*-hexadecane membrane at a mean macroscopic conductance of $10^{-9}$ S was choosen for this calculation. If one uses the single-channel parameters of this system at an ion concentration of $10^{-3}$ M CsCl (see Table II), one obtains a signal-to-noise ratio of about 5; the bandwidth of the recording set up was assumed to be 100 Hz. This value is by a factor of 20 above the corresponding ratio evaluated by the correlator using a commercial noise generator (see Materials and Methods).

Data on the cation selectivity of the gramicidin channel in membranes of different composition are summarized in Table III. It is seen that the cation selectivity is almost independent of hydrocarbon thickness at an ion concentration of 1 M. Also for a lower ion concentration of $10^{-2}$ M CsCl, the cation selectivity does not seem to be significantly changed as can be seen from Table III for monoerucin/*n*-hexadecane membranes.

## Discussion

### *(a) Kinetics of channel formation*

In the framework of the dimerization model of channel formation by gramicidin A the reciprocal value of the dissociation rate constant $k_D$ should be equal to the mean channel life-time $\tau^*$. Comparison of $\tau^*$ as measured by single-channel experiments with $1/k_D$ from relaxation and autocorrelation analysis of bilayer membranes under a variety of conditions shows a close agreement (Table I). The $k_D$ value for monoolein/*n*-hexadecane membranes in 1 M NaCl also agrees with $1/\tau^*$ measured by Hladky and Haydon [3] under slightly different conditions (23°C, 0.5 M NaCl). However, in case of monopalmitolein/*n*-hexadecane membranes our value of $k_D$ obtained by autocorrelation analysis is about ten-times larger than $1/\tau^*$ reported by Hladky and Haydon [3] from single-channel experiments.

Increasing the chain length of the fatty acid of the monoglyceride from $C_{16}$ to $C_{24}$ increases $k_D$ by more than two orders of magnitude (Table I and Fig. 3); at the same time, $k_R$ decreases about ten-fold between $C_{18}$ and $C_{20}$. The variation of the chain length from $C_{16}$ to $C_{24}$ corresponds to a variation in the hydrocarbon thickness $d$ of the membrane from 2.8 to 6.9 nm (with *n*-hexadecane as solvent), or, with a channel length of 1 ~ 3.0 nm [4], to a variation in $1/d$ between 1.1 and 0.4. A strong decrease of the channel life-time $\tau^*$ with increasing $d$ has already been reported by Hladky and Haydon [3]. The results of this study show, however, that $\tau^*$ is not strictly correlated with membrane thickness $d$. This is seen by comparison of the results obtained from monoolein/*n*-hexadecane membranes ($d \simeq 3.2$ nm) with those from solvent-free monoerucin membranes ($d \simeq 3.2$ nm). Despite the fact that the hydrocarbon thickness is approximately the same for both membranes, the values of $k_D \approx 1/\tau^*$ differ by a factor of about 3 (Table I).

Decreasing the ion concentration in the aqueous phase from 1 M to 0.1 M results in an increase of $k_D$ by a factor of about 3 and a decrease of $k_R$ by a factor of 5 (monoolein/*n*-hexadecane membranes; Table I). A smaller effect on $k_D$ is found in the case of monoerucin/*n*-hexadecane membranes. This means that the equilibrium constant $K = k_R/k_D$ of dimerization is appreciably increased at higher ion concentrations. We can only give a tentative explanation for this interesting effect. Increasing the ion concentration $c_M$ increases the probability that the channel is occupied by an ion. The influence of $c_M$ on the dimerization equilibrium could then simply consist in an electrostatic stabilization of the channel (if an ion is located in one half of the dimeric channel, then the replacement of the other half by an equivalent amount of hydrocarbon increases the free energy because the dielectric constant of the hydrocarbon is less than the average dielectric constant of the channel).

### *(b) Single-channel conductance*

In the cases where the channel length is comparable to the thickness of the hydrocarbon core of the membrane, the values of the single-channel conductance obtained by single-channel experiments and autocorrelation analysis are in close agreement. This has been found with monoolein/*n*-hexadecane membranes (Table I) and dioleoyl phosphatidylcholine/*n*-decane membranes [6]; similar results have been reported by Zingsheim and Neher [11]. Hladky and Haydon [3] reported that in a series of membranes of different composition the single-channel conductance was independent of membane thickness $d$. Our measurements with monopalmitolein and monoolein membranes with either *n*-decane or *n*-hexadecane as solvent confirm these observations. Despite a variation of $d$ between 2.2 and 4.8 nm, the single-channel conductance remains virtually constant (Table I). However, with membranes made from monoglycerides with longer chain length $n$, the single-channel conductance strongly decreases with increasing $n$ (increasing thickness), as Table I and Fig. 6 show. The data of Table I further indicate that $\Lambda$ is mainly determined by the nature of the lipid and much less by the solvent used for membrane formation, despite a strong dependence of membrane thickness on the solvent. For instance, the $\Lambda_a$ values of monoerucin membranes formed from $n$-decane solution ($d$ = 5.84 nm), $n$-hexadecane solution ($d$ = 4.85 nm) and solvent-free monoerucin membranes ($d$ = 3.15 nm) are not much different (5.8, 8.0 and 7.8 pS in 1 M NaCl). A similar conclusion holds for monopalmitolein and monoolein membranes in either $n$-decane or $n$-hexadecane.

These findings can be explained by the assumption that a membrane formed from monoglyceride in a hydrocarbon solvent is inhomogeneous with respect to solvent content and thickness [20]. If the membrane contains regions of different thickness, channels may form preferentially in the thinner regions. The properties of such a region which contains less solvent than the average membrane is likely to be determined mainly by the nature of the lipid and much less by the solvent. This conclusion agrees with recent observations on the kinetics of carrier-mediated ion transport in membranes of different composition. A striking feature of the results obtained by autocorrelation analysis is the much stronger decrease of $\Lambda_a$ with increasing hydrocarbon thickness compared with the results of single-channel experiments (Fig. 6). It is seen from Table I that the discrepancy between $\Lambda_a$ and $\Lambda_{sc}$ also occurs for solvent-free membranes. On the other hand, a reduction of ion concentration from 1 M to 0.1 M almost completely removes the difference between $\Lambda_a$ and $\Lambda_{sc}$ (Table I). As the main difference between autocorrelation and single-channel measurements is the larger number of channels in the autocorrelation experiment, a likely explanation of the discrepancy between $\Lambda_a$ and $\Lambda_{sc}$ is interaction between channels. In other words, if this explanation is right then $\Lambda_{sc}$ is the conductance of an isolated channel and $\Lambda_a$ the conductance of a channel within an aggregate of the other channels. An obvious test of this hypothesis would consist in measuring the autocorrelation function at very low channel densities. However, this experiment meets with great difficulties, as the mean membrane current $\bar{J}$ which has to be known for the calculation of $\Lambda$ according to Eqn. 4 cannot be determined with sufficient accuracy during the time period of the measurement. In a few cases, for instance for solvent-free monoerucin membranes, $\bar{J}$ could be estimated in the presence of about 10 to 20 channels within 50% accuracy. The calculation of $\Lambda_a$ from Eqn. 4 led even at this small channel number to the same value of $\Lambda_a$, as given in Table I. With 10—20 channels present in a membrane of about $5 \cdot 10^{-4}$ cm$^2$ of area, the average channel density is too low for an interaction between channels. If, however, a local thinning of the membrane is assumed, a thin region may be stabilized by the presence of a channel and further channels may form preferentially in the vicinity of already existing channels. This would lead to the formation of local clusters of channels. Interactions between channels within such a cluster may occur by mutual perturbation of the geometrical structure of the peptide helices. However, a more likely source of interaction, is the space charge built up by permeating ions. From the dependence of single-channel conductance on ion concentration $c_M$ it may be inferred that at $c_M = 1$ M an appreciable fraction of channels is occupied by an ion [3]. A cation density of about $3 \cdot 10^{-3}$ M in the membrane would result if the channels within the cluster are assumed to have a mean distance of 10 nm and if every channel is occupied by a cation. At this ion density, space-charge effects become rather strong [27], even if it is taken into account that the average dielectric constant of the cluster is higher than that of the unmodified membrane. The hypothesis that the reduction of $\Lambda$ in a multichannel experiment originates from an electrostatic interaction between channels is supported by the finding that the difference between $\Lambda_a$ and $\Lambda_{sc}$ vanishes at lower ion concentrations (Table I).

In addition to the discrepancy between $\Lambda_a$ and $\Lambda_{sc}$ there is also a deviation of the experimental values of $\overline{(\delta J)^2}/\bar{J}^2$ vs. $1/\lambda \cdot A$ from the theoretical curve calculated from Eqn. 3. However, this deviation cannot be explained by an electrostatic effect alone, since it is observed even in the presence of 0.1 M CsCl (Fig. 4). Since the values of $\Lambda$ obtained by the method of correlation analysis are reproducible within about 20%, the local channel density of the membrane must be a rather well defined function of the thickness of the hydrocarbon core. The chain length dependence of the discrepancy of $\Lambda$ could then be explained by an increase of local channel density with increasing membrane thickness.

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