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Dimerization Constant and Single-Channel Conductance of Gramicidin in Thylakoid Membranes

Gerald Schönknecht,* Gerd Althoff, and Wolfgang Junge
Biophysik, Fachbereich Biologie/Chemie, Universität Osnabrück, D-4500 Osnabrück, Federal Republic of Germany

Summary. The effect of the pore-forming antibiotic gramicidin on pure lipid membranes is well characterized. We studied its action in protein-rich thylakoid membranes that contain less than 25% (wt/wt) acyl lipids. A transmembrane voltage was induced by flashing light, and its decay was measured and interpreted to yield the distribution of gramicidin over thylakoids, its dimerization constant and its single-channel conductance in this membrane. The distribution of gramicidin over the ensemble of thylakoids was immediately homogeneous when the antibiotic was added under stirring, while it became homogeneous only after 20 min in a stirred suspension that was initially heterogeneous. The dimerization constant, 5×10^{14} cm^2/mol, was about 10 times larger than in pure lipid membranes. This was attributed to the up-concentration of gramicidin in the small fractional area of protein-free lipid bilayer and further by a preference of gramicidin for stacked portions of the membrane. The latter bears important consequences with regard to bioenergetic studies with this ionophore. As gramicidin was largely dimerized from a concentration of 1 nM (in the suspension) on, the membrane's conductance then increased linearly as a function of added gramicidin. When the negative surface potential at the thylakoid membrane was screened, the conductance of a single gramicidin dimer agreed well with figures reported for bilayers from neutral lipid (about 0.5 pS at 10 mM NaCl). The modulation of the conductance by the surface potential in spinach *versus* pea thylakoids and between different preparations is discussed in detail.

Key Words photosynthesis · thylakoids · electrochromism · gramicidin · conductance · dimerization

Introduction

Gramicidin acts as a transmembrane ion channel for monovalent cations (Hladky & Haydon, 1972; Myers & Haydon, 1972). The conducting channel is formed by two gramicidin monomers that span the membrane as a dimer in a head-to-head configuration (Urry et al., 1971; Bamberg, Apell & Alpes, 1977; Stankovic et al., 1989). The conduction of gramicidin is quantitatively characterized in artificial lipid bilayers. For sodium it follows Michaelis-Menten behavior with a maximum single-channel conductance of 14.6 pS and a K_m = 310 mM (Finkelstein & Andersen, 1981). The potassium conductance is about twofold larger (Hladky & Haydon, 1972; Myers & Haydon, 1972) (at concentrations $< K_m$). The dimerization constant ranges between 10^{11} and 10^{14} cm^2/mol, depending on the lipid composition of the membrane (Bamberg & Läuger, 1973; Veatch et al., 1975).

In bioenergetic studies with mitochondria and chloroplasts gramicidin is widely used. The inner membranes of mitochondria (christae) and chloroplasts (thylakoids) are protein rich (≤30% lipid) and heterogeneous. In thylakoid membranes the conductivity increases linearly as a function of added gramicidin (Junge & Witt, 1968; Schönfeld & Schickler, 1984). This is in seeming contrast to the expectation of a second-order dependence (as the dimer is the conducting species) (Tosteson et al., 1969; Veatch et al., 1975). It has to be questioned whether this was due to complete dimerization or to a different conduction mechanism (monomers in conjunction with other proteins?).

It was the aim of this study to quantify the behavior of gramicidin in the thylakoid membrane. With flash spectrophotometry we determined the conductivity of thylakoid membranes in the range of 10 pM to 1 μM gramicidin. Therefrom we calculated the dimerization constant and single-channel conductance of gramicidin in thylakoid membranes. The results were compared with published data for lipid bilayers.

Materials and Methods

Thylakoids were prepared from pea seedlings (*Pisum sativum* var. "Kleine Rheinländerin") grown in a greenhouse or from spinach leaves (*Spinacea oleracea*) bought on the local market.

* *Present Address:* Lehrstuhl für Botanik I der Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg, FRG.

"Stacked thylakoids" were prepared in the presence of 5 mM $MgCl_2$ as in Polle and Junge (1986). "EDTA-washed thylakoids" were prepared as in Polle and Junge (1986), and "EDTA-vesicles" were prepared as in Lill et al. (1986). EDTA-treated thylakoids were completely destacked (Polle & Junge, 1986), and peripheral proteins including CF_1 (the soluble part of the chloroplast H^+-ATPase) were largely detached (Lill et al., 1986; Schönknecht et al., 1986). While *EDTA-washed thylakoids* comprise about 10^8 chlorophyll molecules, comparable to *stacked thylakoids, EDTA vesicles* are fragmented into smaller units, spherical in shape with 600-nm diameter. They contain only about 5×10^5 chlorophyll molecules (Schönknecht, Althoff & Junge, 1990).

Stock suspensions (2–6 mM chlorophyll) were stored on ice for up to 5 hr before use. Flash spectrophotometric experiments were carried out in a setup as described by Junge (1976) and by Förster, Hong and Junge (1981). Measurements were carried out at room temperature in a cuvette with a 2-cm optical path length and 15-ml volume containing 20 μM chlorophyll. The suspension was excited by short (15-μsec, full width at half maximum) and saturating (1-mJ/cm^2) flashes of red light (wavelength > 610 nm) at 5-sec intervals, and 20–40 transients were averaged. A single-turnover flash of light generates an electrical PD of 30–50 mV across the thylakoid membrane, positive in the lumen, plus an acidification by less than 0.1 pH units in the lumen and an alkalization in the suspending medium (Junge, 1982). The decay rate (k) of the transmembrane voltage, reflecting the membrane's conductance, was measured by electrochromic absorption changes of intrinsic pigments at 522-nm wavelength (Junge & Witt, 1968; Witt, 1979). pH transients in the suspending medium were determined by the absorption changes of phenol red (15 μM) at 559 nm in the absence of added buffers (Junge & Ausländer, 1973). pH transients at the lumenal surface of the thylakoid membrane were measured via neutral red (15 μM plus 2.6 mg/ml bovine serum albumin) at 548 nm (Junge et al., 1979; Hong & Junge, 1983; Junge, Schönknecht & Förster, 1986). In both cases the pH transients were obtained by subtraction of the signal recorded in the absence of the dye from that recorded in its presence. To block proton conductance through exposed CF_0 (the membrane-spanning channel portion of the chloroplast H^+-ATPase), EDTA-treated thylakoids were incubated with 25 μM DCCD[1] for 10 min.

Gramicidin was added to the thylakoid suspension from ethanolic stock solutions (15 to 50 μl) under vigorous stirring (*see below*). The ethanol concentration in the measuring cuvette was held below 0.5%. In the controls without gramicidin, 75 μl of ethanol (corresponding to 0.5%) was added under vigorous stirring. Gramicidin was purchased from Sigma. It contained 88% gramicidin A, 7% gramicidin B and 5% gramicidin C, according to the manufacturer.

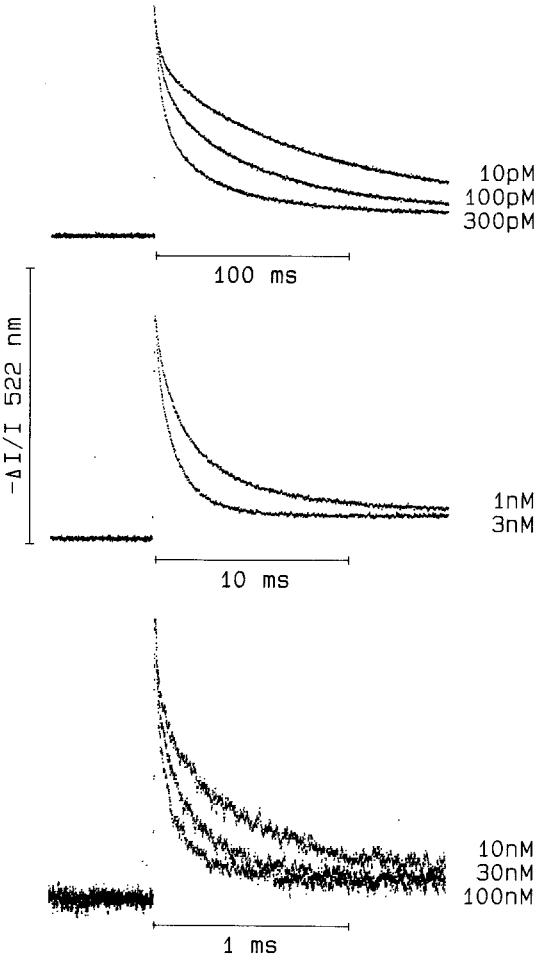

Fig. 1. Time course of the transmembrane electrical potential at eight different gramicidin concentrations, as indicated. Three different time resolutions (time per address of the transient digitizer from top to bottom: 200, 20 and 2 μsec). The vertical bar indicates a relative change of transmitted intensity $-\Delta I/I$, of 7×10^{-3}. *Stacked* pea *thylakoids* in 10 mM KCl, 10 mM $MgCl_2$, 20 μM MV, 5 mM Tris/HCl, pH 7.4.

Results

Records of flash light-induced electrochromic absorption changes at different gramicidin concentrations are shown in Fig. 1 (note different time scales). With increasing concentrations of gramicidin the decay of the transmembrane electrical potential was accelerated, reflecting the added cation conductance. A quantitative evaluation to yield the dimerization constant and the conductance of one gramicidin dimer had to rely on two features: the distribution of gramicidin between water and membranes and the distribution of gramicidin over the thylakoid population.

The Distribution of Gramicidin

Distribution between Thylakoids

Addition of a small volume (typically 15–50 μl) of gramicidin in ethanolic solution to a large volume

[1] *Abbreviations:* DCCD, *N,N'*-dicyclohexylcarbodiimide; MOPS, 3-(N-morpholino)propanesulfonic acid; MV, methylviologen; Tris, Tris(hydroxymethyl)aminomethane; and Chl, chlorophyll.

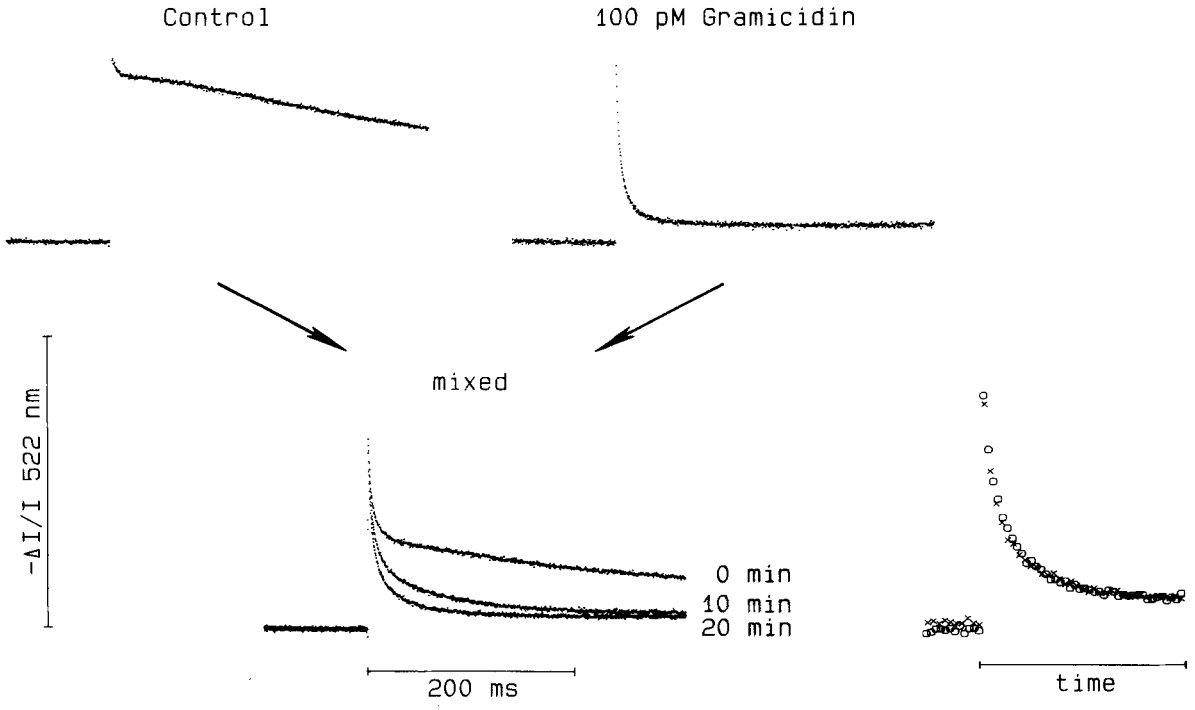

Fig. 2. A test for the homogeneous distribution of gramicidin over thylakoids. A thylakoid suspension containing 100 pM gramicidin (upper right) and a thylakoid suspension without gramicidin (*control*, upper left) were mixed (vol/vol = 1/1) and the electrochromic absorption change was measured once (0 min) and at 10 and 20 min afterwards (bottom). The vertical bar indicates $-\Delta I/I = 7 \times 10^{-3}$. *Stacked* pea *thylakoids* in 10 mM NaCl, 5 mM $MgCl_2$, 10 μM MV, 1 mM MOPS, pH 7.3. Just after mixing the two thylakoid suspensions, a pronounced biphasic decay of the electrochromic absorption change was measured. The slow phase corresponded to the decay kinetics of the control, and the fast phase corresponded to the decay kinetics of the thylakoid suspension containing 100 pM gramicidin. Ten min later the biphasic decay had vanished but it took about 20 min to reach a homogeneous distribution of gramicidin over the thylakoid membranes. This is shown in the lower right: If a thylakoid suspension containing 100 pM gramicidin is mixed with a thylakoid suspension without gramicidin, the gramicidin concentration is halved (50 pM) and the decay of the transmembrane electrical potential under homogeneous distribution of gramicidin is slowed down by a factor of about two. Therefore, the time axis of the electrochromic absorption change of the thylakoid suspension containing 100 pM gramicidin (crosses, the bar corresponds to 50 msec) was expanded by a factor two and compared with the electrochromic absorption change measured 20 min after mixing (circles, the bar corresponds to 100 msec); for both signals the *y*-axis was scaled up by a factor of 1.5.

(15 ml) of a thylakoid suspension might cause an inhomogeneous distribution of gramicidin over thylakoids. We examined the time necessary to reach a homogeneous distribution. A thylakoid suspension which was equilibrated with 100 pM gramicidin was mixed with another one (vol/vol = 1/1) without gramicidin (Fig. 2). Just afterwards, a biphasic decay of the voltage was observed. It reflected the nonhomogeneous initial distribution over thylakoids of gramicidin. The decay became monophasic only after 20 min (*see* Fig. 2). On the other hand, when gramicidin was added to a vigorously stirred thylakoid suspension, the electrochromic absorption changes measured at once and 20 min later showed no significant differences. This indicated that gramicidin was homogeneously distributed right from the beginning.

Distribution between Thylakoids and Aqueous Solution

The pronounced biphasic decay of the electrochromic absorption change just after mixing of the two thylakoid suspensions (without and with 100 pM gramicidin) already pointed out that most of the gramicidin was solved in thylakoid membranes. About 50% of the thylakoids (half the amplitude) showed decay kinetics corresponding to the control (without gramicidin). There was no significant amount of free gramicidin available to accelerate the decay of the transmembrane voltage of the initially gramicidin-free thylakoids. A more quantitative estimate as to which amount of added gramicidin was solved in the thylakoid membranes was based on the following: thylakoids were incubated with various

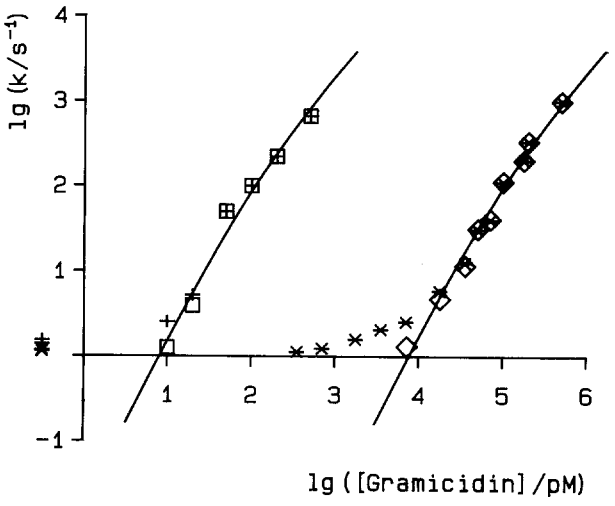

Fig. 3. Distribution equilibrium of gramicidin between the electrolyte solution and the thylakoid membrane. Double logarithmic plot of the decay rates (k/sec^{-1}) of the electrochromic absorption change as a function of the added gramicidin concentration. *Stacked* pea *thylakoids* in 10 mM KCl, 5 mM $MgCl_2$, 20 μM MV, 5 mM MOPS/KOH, pH 7.4. Half-decay time of the control $t_{1/2,C}$ = 775 msec. Data points left of the y-axis represent controls, no gramicidin added. Different gramicidin concentrations were added to thylakoid suspensions, and the electrochromic absorption change was measured (crosses). Different gramicidin concentrations were added to thylakoid suspensions; the thylakoids were spun down by centrifugation; new thylakoids were suspended in the supernatant, and the electrochromic absorption change was measured (asterisks). The measured decay rates were corrected for the decay rate of the control (squares resulting from crosses and rhombs from asterisks) to get the decay rates caused by gramicidin (for details *see* text). The sets of decay rates caused by gramicidin were fitted by Eq. (7), yielding for the direct addition of gramicidin (squares): G_1 = 2.62 pS, K_D = 7.4 × 10^{14} cm^2/mol and for the thylakoids resuspended in the gramicidin containing supernatant (rhombs) G_1 = 2.95 fS, K_D = 7.3 × 10^{11} cm^2/mol. For the latter, the calculated unit conductance of gramicidin (G_1) was lower by a factor of 888 and the dimerization constant (K_D) was lower by a factor of 1014. Taken together, the gramicidin concentration in the supernatant after centrifugation was 950-fold lower than in the thylakoid suspension.

gramicidin concentrations. With one portion of the thylakoid suspension electrochromic absorption changes were measured; the other portion was spun down by centrifugation. New thylakoids were added to the supernatant, and the electrochromic absorption changes were measured. In the latter sample the concentration of gramicidin, which was needed to yield the same acceleration of the decay of the electrical potential as compared to direct addition of gramicidin to thylakoids, was by three orders of magnitude greater (Fig. 3). Accordingly, gramicidin was completely solved in thylakoid membranes, and only about 0.1% remained in the surrounding aqueous solution.

Distribution between Appressed and Nonappressed Regions within a Single Thylakoid

Measurements of the pH inside the thylakoid lumen (pH(lumen)) and of the pH of the suspending medium (pH(medium)) were carried out in the absence and in the presence of gramicidin for *stacked thylakoids* (Fig. 4). The acceleration of the decay of the pH change caused by gramicidin was smaller in the medium than in the lumen (Fig. 4). That is, protons seemed to leak out of the thylakoid lumen (via gramicidin) faster than they arrived in the suspending medium. This was expected if gramicidin was localized in the appressed regions of the thylakoid membrane, as the propagation of a pH pulse is slowed down in the narrow gaps between stacked thylakoid membranes (Junge & Polle, 1986; Polle & Junge, 1986). Corroborating this view, the acceleration of the decay of the pH change in the medium caused by gramicidin was larger for thylakoids destacked by EDTA treatment than for *stacked thylakoids* (*not shown*). Protons reappeared faster in the suspending medium with destacked thylakoids. Measurements of the time dependence of pH changes thus indicated that most gramicidin was solved in the appressed region (grana lamellae) of thylakoids.

Summarizing, gramicidin was completely solved in the membranes and, when added under vigorous stirring, homogeneously distributed over the thylakoid population with a preference for the appressed membrane domains.

Theoretical Consideration

The dimerization of gramicidin which is solved in the membrane behaves as follows (Veatch et al., 1975):

$$K_D = \frac{[D]}{[M]^2} \tag{1}$$

and

$$[T] = [M] + 2[D] \tag{2}$$

with $[M]$, $[D]$, and $[T]$ for the monomer, dimer and total (added) gramicidin concentration (in mol/liter) and the dimerization constant K_D (in liter/mol). In the voltage range ($\leq$50 mV) and time domain ($\leq$100 msec) examined in this paper K_D is constant (Bamberg & Läuger, 1973). The dimer concentration depends on the total gramicidin concentration as given by:

$$[D] = \frac{[T]}{2} + \frac{1 - \sqrt{1 + 8K_D \cdot [T]}}{8K_D}. \tag{3}$$

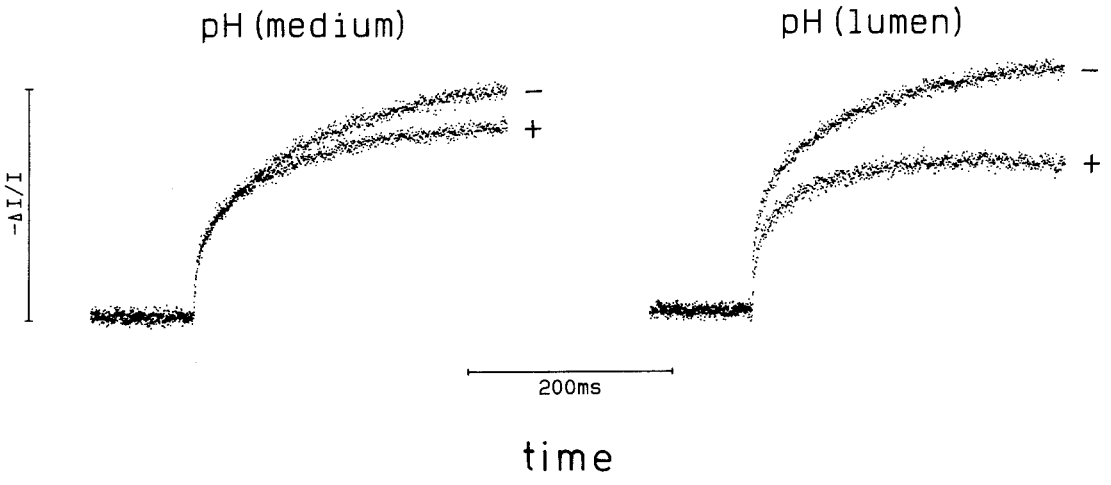

Fig. 4. Time course of the pH-indicating absorption changes in the absence (−) and in the presence of 3 nM gramicidin (+), *stacked* pea *thylakoids*. Left: Absorption changes of phenol red at 559 nm, indicating alkalization of the suspending medium (pH(medium)) after a flash of light. Right: Absorption changes of neutral red at 548 nm, indicating acidification at the lumenal side of the thylakoid membrane (pH(lumen)). The vertical bar indicates a relative change of transmitted intensity $-\Delta I/I$ of 1.9×10^{-3} at 559 nm (pH(medium)) and 1.2×10^{-3} at 548 nm (pH(lumen)). The medium contained 10 mM NaCl, 200 μM $K_3[Fe(CN)_6]$, pH 7.3. The amplitude decrease caused by 3 nM gramicidin reflects the accelerated efflux of protons from the thylakoid lumen via gramicidin into the medium. Three hundred msec after the exciting flash of light the amplitude diminution amounted to 15% as viewed from the medium (left) and 39% as viewed from the lumen (right).

There are two limiting conditions of interest: (i) $K_D \cdot [T] \ll 1$; the total gramicidin concentration is much smaller than the dimerization constant. Most of the gramicidin is monomeric $[M] \cong [T]$. This results in a quadratic dependence of the dimer concentration on the total concentration $[D] \cong K_D \cdot [T]^2$. (ii) $K_D \cdot [T] \gg 1$; most of the gramicidin is dimerized. This results in a linear dependence of the dimer concentration on the total gramicidin concentration $[D] \cong [T]/2$.

The initial slope (or decay rate, k) of the electrochromic absorption change reflects the decay rate (k) of the transmembrane electrical potential which gives the ratio between membrane conductance (G) and capacitance (C):

$$k = \frac{G}{C}. \tag{4}$$

The specific capacitance of the thylakoid membrane, $\hat{C}$, is about 1 μF/cm² (Farkas, Korenstein & Malkin, 1984; Arnold et al., 1985), while the specific conductance, $\hat{G}$, results from:

$$\hat{G} = \frac{G_1 \cdot [D]}{[\text{Chl}] \cdot A_{\text{Chl}}} \tag{5}$$

with the single-channel conductance of gramicidin, G_1 (the conductance of a single gramicidin dimer), the chlorophyll concentration, [Chl] (20 μM), and the chlorophyll density, $1/A_{\text{Chl}}$ (1/2.2 nm², (Wolken & Schwertz, 1953; Stolz & Walz, 1988). So the decay rate (k) of transmembrane voltage depends on the dimer concentration $[D]$ according to:

$$k = \frac{G_1 \cdot [D]}{[\text{Chl}] \cdot A_{\text{Chl}} \cdot \hat{C}}. \tag{6}$$

Combining Eqs. (3) and (6) and inserting constant values yields:

$$\lg(k) = 0.357 + \lg(G_1) + \lg\left(\frac{[T]}{2} + \frac{1 - \sqrt{1 + 8K_D \cdot [T]}}{8K_D}\right). \tag{7}$$

This equation was used to fit sets of decay rates (k in sec^{-1}) of the electrochromic absorption change at various gramicidin concentrations ($[T]$ in pM) to yield the single-channel conductance of gramicidin (G_1 in pS) and the dimerization constant (K_D in pM^{-1}). The K_D was recalculated from volume-related units (1/pmol) into area-related units (cm^2/mol) according to

$$\frac{K_D}{\text{cm}^2/\text{mol}} = \frac{K_D}{1/\text{pmol}} \cdot \frac{A}{V} \cdot 10^{12} \tag{8}$$

with a total membrane area per suspension volume

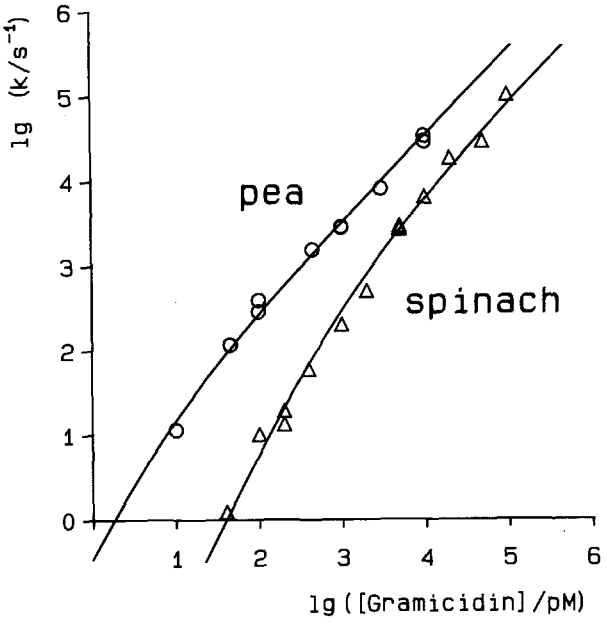

Fig. 5. Double logarithmic plot of the decay rates (k/sec^{-1}) of the transmembrane electrical potential as a function of added gramicidin; comparison of spinach (*S. oleracea*) and pea (*P. sativum*). *Stacked thylakoids* in 10 mM KCl, 20 μM MV, 5 mM Tris/HCl, pH 7.4. Half-decay time of control thylakoids (in the absence of gramicidin), spinach: $t_{1/2,C}$ = 141 msec, pea: $t_{1/2,C}$ = 37 msec. Fit parameters according to Eq. (7), spinach: G_1 = 0.76 pS, K_D = 1.01 × 10^{14} cm²/mol; pea: G_1 = 3.01 pS, K_D = 1.66 × 10^{16} cm²/mol (for details *see* text).

of A/V = 2.65 × 10^5 cm²/liter (1/2.2 nm² and 20 μM Chl).

It is apparent from Eq. (7) that the two fit parameters (G_1 and K_D) are independent from each other. G_1 results from all decay rates (k) and is most reliably determined in the range of the linear dependence of the dimer on the total gramicidin concentration ($[D] \cong [T]/2$, *see above*). K_D results from the concentration range with the transition from the quadratic to the linear dependence.

Dimerization Constant and Conductance

The measured decay rates of the transmembrane potential had to be corrected at very high and very low gramicidin concentrations. At low gramicidin concentrations the extra decay rate caused by gramicidin (k_E) came close to the decay rate of the control (k_C) caused by intrinsic leak conductances. As long as the measured decay rate in the presence of gramicidin was less than 10 times the decay rate of the control, the latter was subtracted from the former ($k = k_E - k_C$) (*see* Fig. 3). At high gramicidin concentrations a limiting maximum decay rate (k_M) was observed. Measured decay rates (k_E) larger than one-tenth of the maximum decay rate were corrected ($k^{-1} = k_E^{-1} - k_M^{-1}$).

Table 1. Dimerization constant of gramicidin*

	K_D (cm²/mol)
Spinach	4.1 (±2.7) × 10^{14} (n = 5)
Pea	
Control: $t_{1/2} \geq$ 300 msec	6.5 (±2.7) × 10^{14} (n = 4)
Control: $t_{1/2} <$ 300 msec	3.9 (±3.7) × 10^{16} (n = 4)
Artificial bilayer	
Phosphatidyl choline	2 × 10^{13} to 1 × 10^{14}
Glycerolester	1 × 10^{11}

* K_D values in spinach and pea thylakoids resulting from fits as shown in Figs. 3, 5, and 6 (number of data sets fitted, n, and SD from the average K_D value given in parentheses) were compared with published K_D values in artificial lipid bilayers (Bamberg & Läuger, 1973; Veatch et al., 1975). The K_D values from pea thylakoids were divided into two groups, as in pea thylakoids with shorter half-decay times of the control ($t_{1/2,C} <$ 300 msec) significantly higher K_D values were observed.

In Fig. 5 two sets of decay rates caused by gramicidin (corrected for k_C and k_M) were fitted according to Eq. (7). The corresponding electrochromic absorption changes were measured under identical conditions with *stacked thylakoids* of two different plant species, spinach (*S. oleracea*) and pea (*P. sativum*). The resulting fit parameters were: for spinach, G_1 = 0.8 pS, K_D = 1 × 10^{14} cm²/mol; for pea, G_1 = 3 pS, K_D = 1.7 × 10^{16} cm²/mol. The single-channel conductance of gramicidin (G_1) as well as the dimerization constant (K_D) varied with thylakoids from different plant species (Fig. 5).

Table 1 summarizes K_D values from spinach and pea thylakoids for different thylakoid preparations and compares them with those from artificial lipid bilayers. The dimerization constant of gramicidin in thylakoid membranes was about an order of magnitude larger than in artificial lipid bilayers. For spinach thylakoids the K_D value (4.1 ± 2.7 × 10^{14} cm²/mol) did not vary depending on the thylakoid preparation or the "intactness" of the membrane, as evident from the half-decay time of the control. For pea thylakoids the dimerization constant (K_D) was related to the thylakoid preparation. Concomitant with less leaky membranes, as evident from longer half-decay times of the control ($t_{1/2,C} >$ 300 msec), the K_D values were comparable to those from spinach (6.5 ± 2.7 × 10^{14} cm²/mol; *see* Fig. 3, very "intact" *stacked* pea *thylakoids*, $t_{1/2,C}$ = 775 msec and K_D = 7.4 × 10^{14} cm²/mol). But at shorter half-decay times of the control ($t_{1/2,C} <$ 300 msec) the K_D values were about 100-fold larger (3.9 ± 3.7 × 10^{16} cm²/mol; *see* Fig. 5, *stacked* pea *thylakoids* with $t_{1/2,C}$ = 37 msec and K_D = 1.7 × 10^{16} cm²/mol).

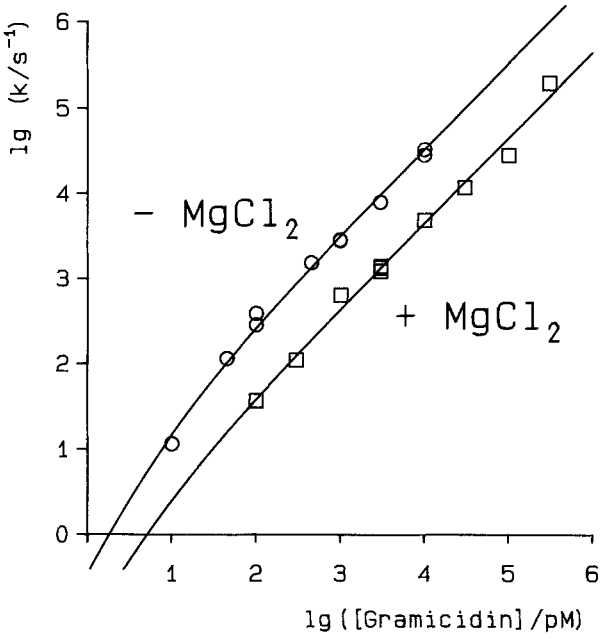

Fig. 6. Double logarithmic plot of the decay rates (k/sec^{-1}) of the transmembrane electrical potential as a function of added gramicidin, without $MgCl_2$ (circles, same data points as in Fig. 5) and in the presence of 10 mM $MgCl_2$ (squares, data points from curves in Fig. 1). *Stacked* pea *thylakoids* in 10 mM KCl, 20 μM MV, 5 mM Tris/HCl, pH 7.4. Half-decay times of control thylakoids, without $MgCl_2$: $t_{1/2,C}$ = 37 msec, with $MgCl_2$: $t_{1/2,C}$ = 45 msec. Fit parameters, without $MgCl_2$: G_1 = 3.01 pS, K_D = 1.66 × 10^{16} cm^2/mol; with 10 mM $MgCl_2$: G_1 = 0.40 pS, K_D = 3.34 × 10^{16} cm^2/mol (for details *see* text).

We suspected that the different single-channel conductances of gramicidin (G_1) in pea and in spinach thylakoids were caused by different cation activities in the thylakoid lumen. This was investigated via the effect of added Mg^{2+} which is supposed to modulate the surface potential and thereby the surface concentration of monovalent cations. Figure 6 shows the decay rates caused by gramicidin in the absence and presence of $MgCl_2$ (10 mM). The fit parameters underlying the lines were calculated according to Eq. (7): without $MgCl_2$, G_1 = 3 pS, K_D = 1.7 × 10^{16} cm^2/mol and with $MgCl_2$, G_1 = 0.4 pS, K_D = 3.3 × 10^{16} cm^2/mol. While the dimerization constant (K_D) was changed by less than a factor of two by the addition of 10 mM $MgCl_2$, the single-channel conductance of gramicidin (G_1) decreased by more than a factor of seven. So the single-channel conductance of gramicidin (G_1) for K^+ in pea thylakoids in the presence of $MgCl_2$ was nearly the same as in spinach thylakoids in the absence of $MgCl_2$.

We repeated the experiments on the effects of $MgCl_2$ with different thylakoid preparations from both spinach and pea for K^+ as well as Na^+ as permeating cation. The resulting single-channel conductances (G_1) are summarized in Table 2. The effects of $MgCl_2$ were more pronounced in pea thylakoids than in spinach thylakoids. In the former, the single-channel conductance was decreased by up to a factor of seven by the addition of $MgCl_2$ (*see* Fig. 6), while Mg^{2+}-ion depletion by EDTA increased the single-channel conductance two to threefold (*EDTA-washed thylakoids* and *EDTA vesicles* compared to *stacked thylakoids*). So the single-channel conductance of gramicidin in pea thylakoids (for 10 mM Na^+ or K^+) could be changed by up to one order of magnitude by Mg^{2+}-ion depletion or addition. For *stacked* pea *thylakoids* the effect of $MgCl_2$ on G_1 was related (as the K_D values) to the intactness of the thylakoid membrane. At longer decay times of the control ($t_{1/2,C}$ > 300 msec), addition of $MgCl_2$ hardly decreased the single-channel conductance (for a preparation with $t_{1/2,C}$ = 450 msec, G_1 decreased only from 1.0 pS (−$MgCl_2$) to 0.85 pS (+$MgCl_2$) in 10 mM NaCl). At shorter half-decay times of the control, the effect of $MgCl_2$ on the single-channel conductance became stronger (*see* Fig. 6). Therefore in Table 2, for *stacked* pea *thylakoids* in the presence of $MgCl_2$, the range of observed G_1 values was given.

Summarizing the data from Table 2, three features, besides the $MgCl_2$ effects, became evident: (i) The single-channel conductance of gramicidin for K^+ was about twofold larger than the single-channel conductance for Na^+. This was in accordance with data from the literature (Hladky & Haydon, 1972; Myers & Haydon, 1972). (ii) The single-channel conductances of gramicidin calculated from flash spectrophotometric measurements with thylakoids in the presence of $MgCl_2$ (Table 2) agreed well with published data for the single-channel conductance of gramicidin based on electrophysiological measurements with artificial bilayer membranes from neutral lipids (10 mM Na^+: 0.46 pS (Finkelstein & Andersen, 1981)). (iii) At otherwise comparable conditions, the single-channel conductance of gramicidin in spinach thylakoids was significantly lower than in pea thylakoids.

Discussion

We showed that gramicidin was completely solved in thylakoid membranes with a preference for the appressed regions. It was homogeneously distributed over the thylakoid population. Homogeneity was achieved by vigorous stirring of the thylakoid suspension when small volumes of gramicidin in ethanol were added. Otherwise it took some 10 min for an initially heterogeneous distribution of gramicidin to be dissipated.

We measured the decay rate of the transmem-

Table 2. Single-channel conductance (G_1 in pS) of gramicidin in thylakoid membranes*

	$-MgCl_2$ (10 mM NaCl)	$+MgCl_2$ (10 mM NaCl)	$-MgCl_2$ (10 mM KCl)	$+MgCl_2$ (10 mM KCl)
Spinach				
Stacked thylakoids	0.5 (2)	0.4 (2)	1.0 (2)	0.6 (2)
EDTA vesicles	1.5	1.0	3.0	—
Pea				
Stacked thylakoids	1.2 (±0.3) (5)	0.3–0.9 (7)	3.0	0.4–2.6 (3)
EDTA-washed thylakoids	2.3	—	—	—
EDTA vesicles	3.4 (±0.6) (5)	1.8 (2)	6.5 (2)	3.3

* G_1 values from different spinach and pea thylakoid preparations at various ionic compositions. In parentheses are the number of data averaged (SD are given for only two values). For *stacked* pea *thylakoids* in the presence of $MgCl_2$, instead of an average the range of observed values is given, as there was a significant dependence of the observed G_1 values on the half-decay time of the control (for details *see* text).

brane voltage as a function of the gramicidin concentration and, on the basis of a homogeneous distribution over the thylakoid population, calculated the single-channel conductance (G_1) and the dimerization constant (K_D) of gramicidin (Eq. (7)) in thylakoid membranes. Compared to artificial lipid bilayers, the dimerization constant of gramicidin (K_D) in thylakoid membranes was about one order of magnitude larger (Table 1). This can be rationalized on the basis of the molecular composition of the thylakoid membrane. About 60% of the thylakoid membrane is occupied by protein, and about 50% of the lipid is in direct contact with protein forming a monomolecular annulus (Murphy, 1986). So the area remaining for free diffusion of gramicidin covers only about 20% of the total area of the thylakoid membrane. Additionally gramicidin was mostly solved in the appressed regions in stacked thylakoids (Fig. 4). If concentrated in the lipid domains of the appressed regions the effective gramicidin concentration in this area is 5- to 10-fold larger compared to a uniform distribution of gramicidin over the entire thylakoid membrane. Correspondingly, if gramicidin covers only 10–20% of the total membrane area the K_D value (in cm^2/mol) is reduced by a factor of 5–10 according to Eq. (8). The so-corrected K_D values of gramicidin in thylakoid membranes were of the same order of magnitude as published K_D values in artificial lipid bilayers.

The upconcentration of gramicidin in the appressed regions of stacked thylakoids bears consequences for the interpretation of bioenergetic studies with gramicidin. In the appressed regions gramicidin is close to photosystem II but relatively far from photosystem I and the H^+-ATPase. Together with lateral resistances for proton diffusion within the thylakoid lumen (Haraux & de Kouchkovsky, 1983; Junge & Polle, 1986) this resulted in a more effective uncoupling by gramicidin of photosystem II-driven ATPsynthesis than photosystem I-driven ATPsynthesis (G. Schönknecht & W. Junge, *in preparation*).

The reason for the 100-fold larger dimerization constant in those preparations of pea thylakoids which initially revealed shorter half-decay times (Table 1) is not understood. It is known that the rate constant of channel formation (gramicidin dimerization) is several orders of magnitude less than the limiting value for a diffusion-controlled reaction (Bamberg & Läuger, 1973). There is a dependence of the rate constant of channel formation upon the lipid composition of the membrane, but the underlying mechanisms are not understood (*see* Andersen, 1984). The average single-channel lifetime also depends on the membrane composition. In this case there is a good correlation with the membrane surface tension, with a shorter channel lifetime as the tension increases (Neher & Eibl, 1977), although membrane thickness may also play a role (*see* Andersen, 1984). To our knowledge there are no particular properties of the membrane composition in pea thylakoids which may be correlated with the observed 100-fold increase of the dimerization constant of gramicidin.

Earlier studies of the conductivity of thylakoid membranes have revealed a linear increase as a function of added gramicidin (Junge & Witt, 1968; Schönfeld & Schickler, 1984). This observation, which was in seeming contradiction with the dimer being the channel-forming species, is now understood. It is due to the relatively large dimerization constant of gramicidin in thylakoid membranes. As the thylakoid preparations used in the earlier studies had larger intrinsic conductances (lower $t_{1/2,C}$) at least 1 nM gramicidin (at 10 μM chlorophyll) had to be added before an increase of the membrane conductance

was detectable. With 1 nM gramicidin at 10 μM chlorophyll the specific gramicidin concentration is 7.5×10^{-15} mol/cm^2 (2.2 nm^2 per chlorophyll molecule and complete binding of gramicidin assumed, *see above*). With $K_D = 5 \times 10^{14}$ cm^2/mol (*see* Table 1) 35% of gramicidin is dimerized according to Eq. (3) at this concentration. So the older experiments were carried out in a concentration range where gramicidin was largely dimerized, and this resulted in the linear concentration dependence.

The flash spectrophotometric experiments yielded the conductance of a single gramicidin dimer (G_1, *see* Eq. (5)). As the gramicidin dimer is responsible for the open state of this channel (Urry et al., 1971; Bamberg et al., 1977; Stankovic et al., 1989) G_1 was rightly termed "single-channel conductance" throughout this paper. It is the same as the conductance of the open state as measured by electrophysiological techniques (Table 2).

The effect of $MgCl_2$ on the single-channel conductance of gramicidin in thylakoid membranes pointed to a modulation of the surface concentration of monovalent cations inside the thylakoid lumen. A direct interaction of Mg^{2+} ions and gramicidin is not expected for the concentrations applied here ($\leq$10 mM) (Bamberg & Läuger, 1977). A single-turnover flash of light generates a transmembrane electrical potential that drives cations out of the thylakoid lumen. The single-channel conductance of gramicidin is determined by the surface concentration of monovalent cations inside the thylakoid. A surplus of fixed negative charges inside the thylakoid lumen causes the enrichment of cations at the channel mouth. In an artificial bilayer of *neutral lipids* in 10 mM Na^+, the single-channel conductance of gramicidin is 0.46 pS (Finkelstein & Andersen, 1981). The conductance is about twofold larger in 10 mM K^+ (Hladky & Haydon, 1972; Myers & Haydon, 1972). These values correspond to the single-channel conductances observed in *stacked thylakoids* in the *presence* of $MgCl_2$ (Table 2). When $MgCl_2$ was omitted from the thylakoid suspension to unscreen the surface charges (*stacked thylakoids* prepared in the presence of $MgCl_2$ but suspended in the absence of $MgCl_2$), the single-channel conductance of gramicidin increased accordingly. If thylakoids were even further Mg^{2+} depleted by incubation with EDTA (*EDTA-washed thylakoids* and *EDTA vesicles*) a further increase was observed (Table 2). The smaller increase of the single-channel conductance in spinach thylakoids upon Mg^{2+} depletion (Table 2) may be explained by a lower concentration of fixed negative charges at the lumenal interface of the membrane.

At concentrations of some 10 mM monovalent cations the single-channel conductance of gramicidin is quasi-linearly related to the cation concentration (*see above;* Finkelstein & Andersen, 1981). So the increase of the single-channel conductance by one order of magnitude by Mg^{2+} depletion in pea thylakoids indicated a 10-fold enhancement of the surface concentration of monovalent cations. This corresponded to a surface potential of -60 mV and, according to the Gouy-Chapman model, a surface charge density (at 10 mM NaCl or KCl) of -1.7 μC/cm^2 or 1 e^-/9 nm^2 or 1 e^-/5 Chl molecules (McLaughlin, 1977). This surface charge density compared well with figures obtained by other methods which range from -1.0 μC/cm^2 (Itoh, 1979) for the environment of photosystem I to -3 μC/cm^2 (Yerkes & Babcock, 1981) for the environment of photosystem II. In destacked thylakoids the lateral segregation of membrane proteins changes into a homogeneous distribution and the surface charge density is expected to be homogenized and thereby averaged, accordingly.

In *stacked thylakoids* with long half-decay times of the voltage ($t_{1/2} > 300$ msec) the single-channel conductance of gramicidin was hardly modulated by $MgCl_2$. This is in accordance with earlier observations. In fresh thylakoids prepared in the presence of $MgCl_2$, the lumen is inaccessible for charged buffer molecules (Flores, Graan & Ort, 1983; Hong & Junge, 1983) and the surface potential is insensitive to the addition of divalent cations (Hong & Junge, 1983). In these "very intact" thylakoids the lumenal ion composition seems to be "clamped" and is hardly influenced by the external medium.

Very recently Nishio and Whitmarsh (1991) have studied the conductance of gramicidin in thylakoid membranes. The gramicidin concentration required for collapsing the transmembrane electrical potential (as indicated by the flash-induced electrochromic absorption change) in spinach thylakoids (and intact chloroplasts) has been determined. Nishio and Whitmarsh (1991) have observed that "gramicidin from a higher concentration stock solution was less effective than from a lower concentration stock solution." In other words, the greater the added volume containing the same amount of gramicidin the greater the effect. According to our work, this is the typical outcome when there is no sufficient intermixing during the addition of gramicidin. It results in a heterogeneous distribution of gramicidin over the thylakoid population. Accordingly these authors need more than 10-fold higher gramicidin concentrations to induce the same acceleration than we needed in this work.

In conclusion, in thylakoids from pea or spinach the dimerization constant of gramicidin is larger than in lipid membranes probably because this antibiotic is up-concentrated in those membrane domains with

lipid bilayer character. There the single-channel conductance of gramicidin is in the range observed in neutral lipid bilayers, when the enrichment of alkali cations caused by the negative surface potential is taken into account.

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