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# Temperature-Jump and Voltage-Jump Experiments at Planar Lipid Membranes Support an Aggregational (Micellar) Model of the Gramicidin A Ion Channel

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**Summary.** The kinetics of formation and dissociation of channels formed by gramicidin A and two analogues in planar lipid membranes was studied using a laser temperature-jump technique developed earlier [Brock, W., Stark, G., Jordan, P.C. (1981), *Biophys. Chem.* **13**:329–348]. The time course of the electric current was found to agree with a single exponential term plus a linear drift. In case of gramicidin A the relaxation time was identical to that reported for V-jump experiments [Bamberg, E., Läuger, P. (1973), *J. Membrane Biol.* **11**:177–194], which were interpreted on the basis of a dimerization reaction. The same results were obtained for gramicidin A and for chemically dimerized malonyl-bis-desformylgramicidin. It is therefore suggested that the dimerization represents a parallel association of two dimers to a tetramer. There is evidence that the tetramer, contrary to the presently favored dimer hypothesis, is the smallest conductance unit of an active gramicidin channel. An additional V-jump-induced relaxation process of considerably larger time constant is interpreted as a further aggregation of gramicidin dimers.

**Key Words** gramicidin A · ion channel · lipid membrane · temperature jump · voltage jump

## Introduction

The linear pentadecapeptide GA (gramicidin A),[1] a metabolite of *Bacillus brevis*, is generally accepted as a model for ion channels. Biological membranes [18, 19, 36] and also artificial lipid membranes [31, 33, 45] become selectively permeable for small monovalent cations in the presence of this substance. There are only relatively small differences between alkali ions such as $Na^+$ and $K^+$ [35]. This is contrary to the depsipeptide valinomycin or the macrotetrolide nonactin, which discriminate between $K^+$ and $Na^+$ by many orders of magnitude [44]. Sufficient evidence is obtained from many experimental techniques and strategies (*see* e.g. references [30] or [44] for a review) that these compounds act as mobile ion carriers. Gramicidin A on the other hand is generally thought to form ion channels in membranes. The main support for a channel mechanism is obtained from single-channel measurements [22, 23]. The transport rates derived from these studies are larger by several orders of magnitude than those estimated for ion carriers from voltage-jump relaxation experiments [30, 43].

The primary structure of the polypeptide gramicidin A [39] consists almost exclusively of strongly hydrophobic amino acids in an alternating L-D sequence. The molecule is electrically neutral. Its N-terminal valine is blocked by a formyl group and its C-terminal tryptophane by an ethanolamine residue. Two different molecular models of the gramicidin A channel have been proposed. Both of them assume that the channel is formed by a helical dimer. Urry [46–48] proposed two left-handed, single-stranded $\pi^6_{L,D}$ helices dimerized by head-to-head (formyl end to formyl end) hydrogen bonding as the dominant channel structure. A similar helix for peptide chains containing L and D residues was also suggested by Ramachandran and Chandrasekaran [37]. Veatch et al. [49–51] described parallel and antiparallel double-stranded $\beta$ helices for gramicidin A in organic solvents of different polarity. Both types of helical structures allow ion permeation through a central hole along the helix axis, which is lined with the peptide C–O groups, while the hydrophobic residues represent the exterior surface of the channel in contact with the membrane.

Different experimental strategies have shown that a dimerization process is, indeed, essential for channel activity in lipid membranes. It was found that the membrane conductance increases with the second power of the gramicidin concentration in water [2, 45]. A similar relation was reported for the conductance as a function of the fluorescence (indicating the membrane concentration) of a dansylated gramicidin analogue [52]. The kinetics of channel formation measured either by voltage-jump relaxation experiments [6] or by noise analysis [29, 55]

[1] Abbreviations: GA = gramicidin A, OPG = O-pyromellitylgramicidin A, MBDG = malonyl-bis-desformylgramicidin.

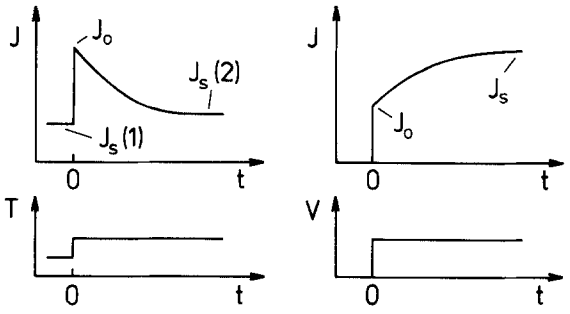


**Fig. 1.** Schematic illustration of the relaxation of the electric current induced by a T jump and a V jump in the presence of gramicidin A and analogues

were also in agreement with the requirements of a dimerization reaction. Finally, hybrid channels are observed, if single-channel experiments are performed in the presence of different gramicidin analogues [2, 32, 53].

Bamberg et al. [3] used gramicidin analogues chemically modified either at the C-terminal or the N-terminal end of the peptide chain, to discriminate between the model proposed by Urry [46–48] and the model suggested by Veatch et al. [49–51]. The measurements were in favor of the $\pi_{L,D}$ helix. The latter represents the model of the gramicidin A channel which is in agreement with all currently available experimental data.

Though there is abundant evidence for a dimerization reaction (*see above*), a definite conclusion about the number of gramicidin A monomers within an active ion channel has not been achieved. A critical survey of the results so far reported (*see* Discussion) shows that the dimerization may be interpreted either as an association of two gramicidin monomers to a dimer or alternatively as an aggregation of two preformed dimers to a tetramer. The possibility of dimer-dimer interactions was first discussed by Urry [47] on the basis of his conformational studies. Direct evidence for a dimer aggregation has recently been obtained by different methods for gramicidin A incorporated into lysolecithin micelles [14, 20, 42]. We have examined the kinetics of channel formation in planar lipid membranes with our recently developed laser T-jump technique. An identical behavior was found for gramicidin A and for chemically dimerized malonyl-bis-desformylgramicidin. As a consequence we suggest that the smallest conductance unit of an active gramicidin channel is a tetramer. The dimerization is interpreted as an association of two dimers to a tetramer.

## Materials and Methods

The experiments were performed on black lipid membranes formed from a 1% solution of 1,2-dioleoyllecithin (Avanti or Sigma) in *n*-decane (Fluka, puriss.). Unbuffered aqueous solutions of 1 M NaCl (pH about 6) were used throughout. Commercially available gramicidin (a mixture of gramicidin A, B and C at approximate ratios 8 : 1 : 2) was dissolved in methanol and kept as a stock solution at −20°C. Trace amounts of this solution were added to the aqueous solutions in which the membranes were prepared. The methanol content of the aqueous solutions did not exceed the 1% level. In a similar way aqueous solutions were prepared containing either the chemically dimerized analogue malonyl-bis-desformylgramicidin synthesized by K. Janko [5] or O-pyromellitylgramicidin, a negatively charged gramicidin analogue synthesized by H. Alpes [2].

The laser T-jump method as applied to planar lipid membranes was described in a previous publication [12]. In brief, the temperature of the membrane and the surrounding aqueous phase is increased by a few tenths of a degree (typically 0.2 to 0.3°C) by absorption of a high-intensity infrared flash (wavelength 1.06 $\mu$m, width 400 $\mu$sec), which is produced by an Nd-glass laser (JK Lasers Ltd., GB). The response of the electric current is observed at constant applied voltage. The temperature remains constant up to several seconds after the flash. At longer times there is a temperature decrease due to heat conduction and convection. The time interval of constant temperature depends on the diameter of the laser beam which was varied by an aperture. At relatively large apertures (maximum diameter of the beam 18 mm), the results may be influenced by artifacts. It was found that the interaction of shock waves, produced by the fast heating, with the Teflon® support surrounding the membrane, can lead to mechanical distortions of the membrane. These are detected by capacitive changes, which may modify the relaxation behavior of the membrane. Artifacts can be avoided by using smaller apertures. Then, however, the temperature stability is impaired. We usually used membranes with a diameter of 2 to 3 mm, and beam apertures of 10 mm. This was sufficient to avoid artifacts and, simultaneously, provided good temperature stability. More than 90% of the original temperature-jump amplitude was observed after 4 sec, and more than 85% after 8 sec. Membrane current was converted to voltage (low-noise operational amplifier Burr-Brown, model OPA 102), amplified by Tektronix oscilloscope 5103 N/5A22N and fed into a transient recorder (Biomation model 805, 2048 words memory size, 8 bits resolution). The data were analyzed with a Hewlett Packard calculator, model HP85. Marquardt's gradient-expansion algorithm [9] was used to fit the data by appropriate mathematical functions.

The voltage-jump method was applied as described previously [30, 43]. The detection and analysis of the relaxation signal was performed as described for the T-jump method. The initial current $J_o$ (*see* Fig. 1) was suppressed to measure relaxation processes of small amplitude with increased gain. The time ranges of the two relaxation processes observed in the presence of gramicidin and its analogues differed by at least one order of magnitude. Therefore, the dual time base of the transient recorder was used to increase the sample interval after the completion of the faster process. In this way both processes could be measured within one experiment. The measurement time of a voltage-jump experiment was about 200 sec. Sufficient time (>20 min) was permitted to restore the initial state before a further experiment was started. All experiments were performed with

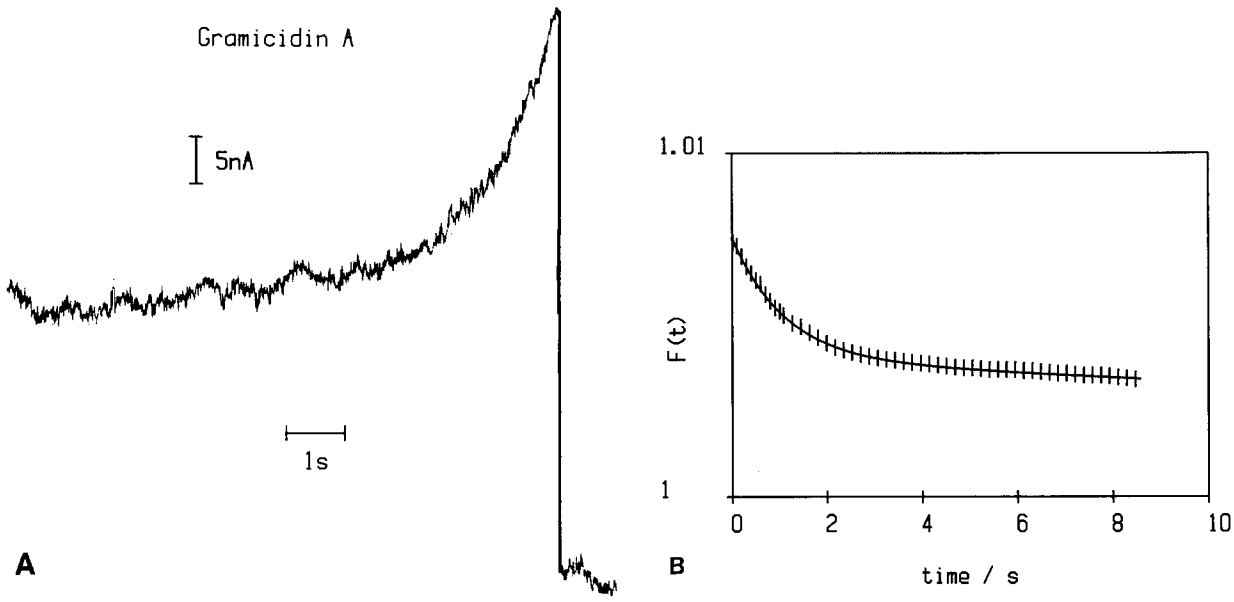


**Fig. 2.** (*A*) Typical relaxation of the electric current induced by a T jump in the presence of gramicidin A. The conductance of the membrane was $10^{-2}\,\Omega^{-1}\,cm^{-2}$; the applied voltage was 50 mV (time axis right to left). (*B*) Fit of the data by Eq. (1). The center of the bars represent the data used for the fitting procedure. The length of the bars indicate the measurement uncertainty. The current was corrected for the resistance of the aqueous phase in series to the membrane (*cf.* Appendix A). Instead of $J(t)$, the reduced current $F(t) = J(t)/J_s(1)$ was plotted. Values of the parameters: $J_s(1) = 5.1 \times 10^{-4}$ A/cm$^2$, $F_s(2) = 1.004$, $\tau_T = 1.11$ sec, $\alpha_T = 4.22 \times 10^{-3}$, $\beta_T = 3.28 \times 10^{-8}$ A/sec

"aged" membranes, which were in the black state for at least 30 min. The temperature was 20°C.

## The Results and their Interpretation

The response of the membrane current found after a T- or V-jump in the presence of gramicidin A is schematically illustrated in Fig. 1. A sudden increase of the steady-state current $J_s(1)$ is observed at a T-jump, followed by a decrease towards a new steady-state $J_s(2)$. The stepwise change $J_s(1) \rightarrow J_o$ is believed to reflect the temperature dependence of the conductance of the active channels existing in state 1 (i.e. at temperature $T$). The decrease $J_o \rightarrow J_s(2)$ is interpreted as an adaptation of the number of active channels to the enhanced temperature $T + \Delta T$ (state 2). In a similar way, following Bamberg and Läuger [6], the V-jump-induced relaxation of $J$ is believed to reflect a voltage-induced increase of active channels. The current $J_o$ corresponds to the number of channels at $V = 0$, while $J_s$ corresponds to the state of the membrane at the voltage $V$.

Figure 2*A* shows the record of a typical T-jump-induced relaxation in the presence of gramicidin A. The steady-state level $J_s(2)$ is not observed. Following a steep decay there is a further small but continuous decrease of $J$. The data (*cf.* Fig. 2*B*) within the experimental error agree with the function

$$J(t) = J_s(2)(1 + \alpha_T \exp(-t/\tau_T)) - \beta_T t. \quad (1)$$

The exponential term is explained below on the basis of the formation and dissociation of active channels. The linear term $-\beta_T$ is a correction term, which was introduced to account for the small decrease of the temperature found in the range of a few seconds after the T jump (*see* Materials and Methods). A second relaxation process observed with the V-jump technique (*see below*) could also contribute to this term. Equation (1) contains the relaxation time $\tau_T$, the relaxation amplitude $\alpha_T$ and the drift correction constant $\beta_T$ as free parameters, which were adjusted to provide optimum fit of the data. The steady-state current $J_s(2)$ is obtained from the relation

$$J_s(2) = J_o/(1 + \alpha_T). \quad (2)$$

Information on the molecularity of chemical reactions is usually obtained from the concentration dependence of the relaxation parameters. Therefore, measurements were performed at different channel

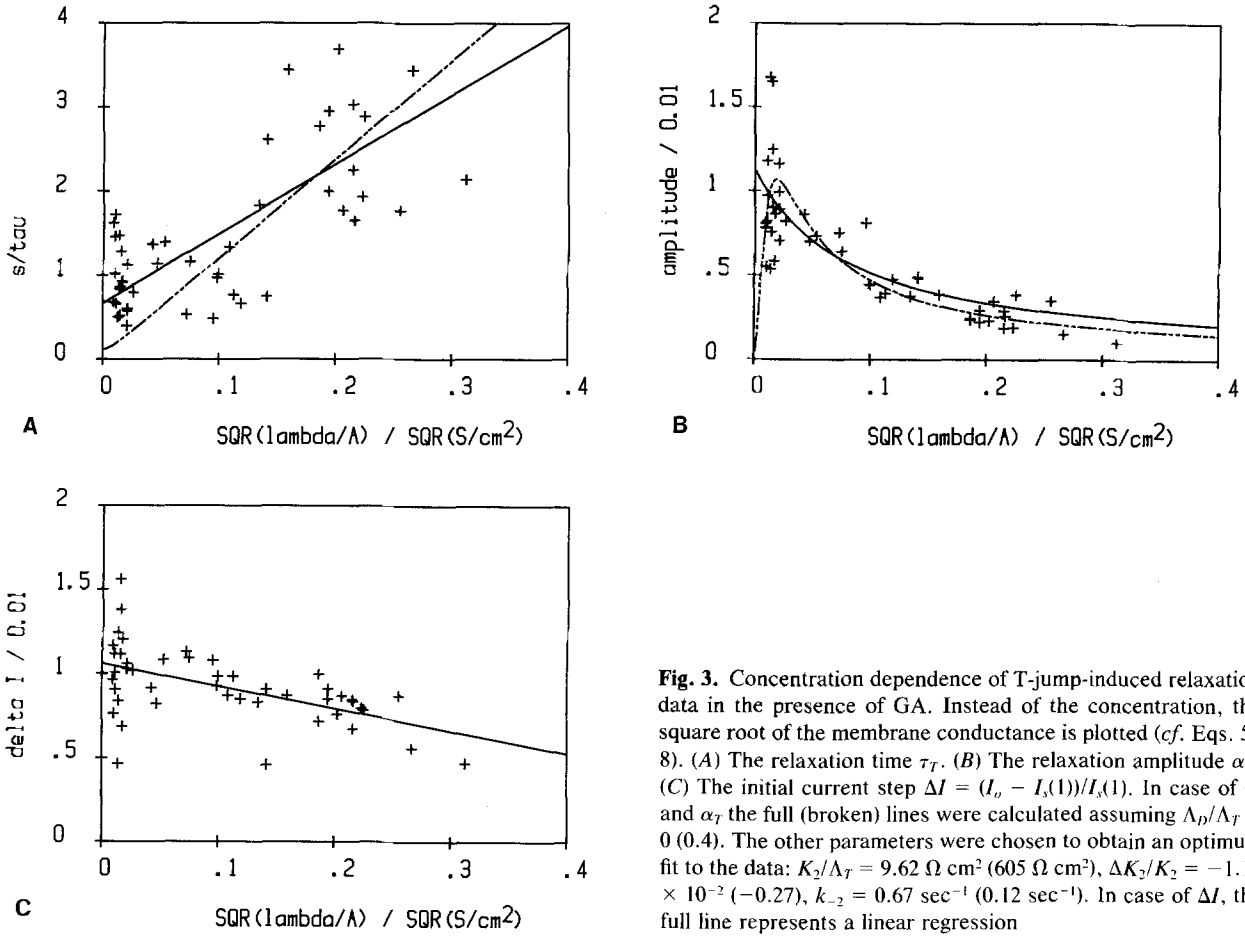


**Fig. 3.** Concentration dependence of T-jump-induced relaxation data in the presence of GA. Instead of the concentration, the square root of the membrane conductance is plotted (*cf.* Eqs. 5–8). (*A*) The relaxation time $\tau_T$. (*B*) The relaxation amplitude $\alpha_T$. (*C*) The initial current step $\Delta I = (I_o - I_s(1))/I_s(1)$. In case of $\tau_T$ and $\alpha_T$ the full (broken) lines were calculated assuming $\Lambda_D/\Lambda_T = 0$ (0.4). The other parameters were chosen to obtain an optimum fit to the data: $K_2/\Lambda_T = 9.62\ \Omega\ \text{cm}^2$ ($605\ \Omega\ \text{cm}^2$), $\Delta K_2/K_2 = -1.12 \times 10^{-2}$ ($-0.27$), $k_{-2} = 0.67\ \text{sec}^{-1}$ ($0.12\ \text{sec}^{-1}$). In case of $\Delta I$, the full line represents a linear regression

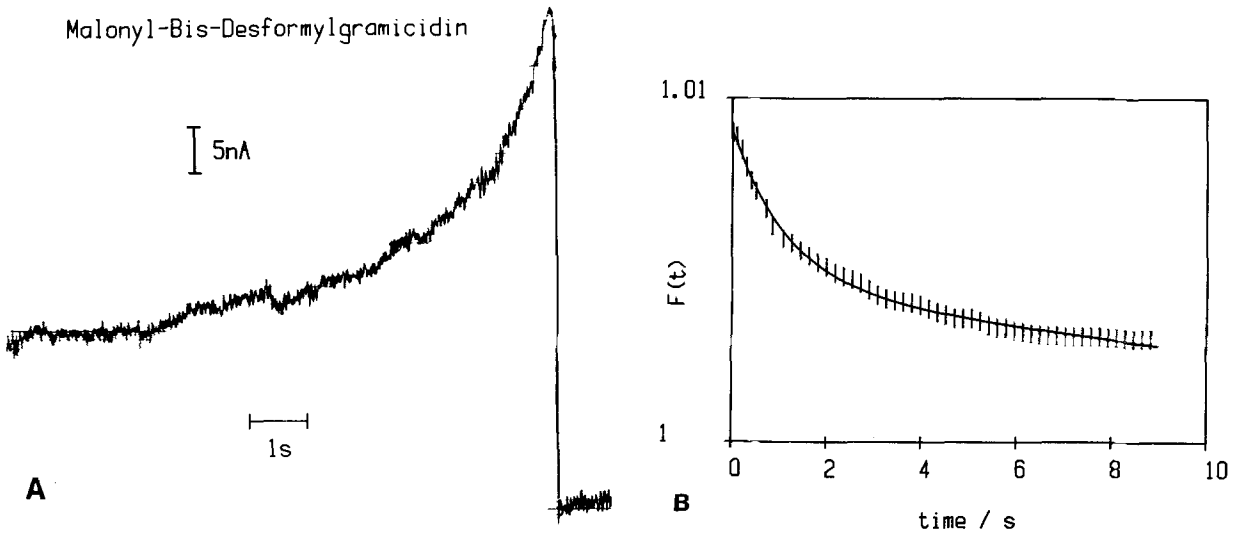


**Fig. 4.** (*A*) Typical relaxation of the electric current observed after a T jump in the presence of chemically dimerized malonyl-bis-desformylgramicidin. The conductance of the membrane was $6.9 \times 10^{-3}\ (\Omega\ \text{cm}^2)^{-1}$ at an applied voltage of 50 mV (time axis right to left). (*B*) Fit of the data by Eq. (1). The center of the bars represent the data used for the fitting procedure. The length of the bars indicates the measurement uncertainty. The current was corrected for the resistance of the aqueous phase in series to the membrane (*see* Appendix A). Instead of $J(t)$, the reduced current $F(t) = J(t)/(J_s(1))$ was plotted. Values of the parameters: $J_s(1) = 3.44 \times 10^{-4}$ A/cm$^2$, $F_s(2) = 1.00445$, $\tau_T = 1.2$ sec, $\alpha_T = 5.31 \times 10^{-3}$, $\beta_T = 6.27 \times 10^{-9}$ A/sec

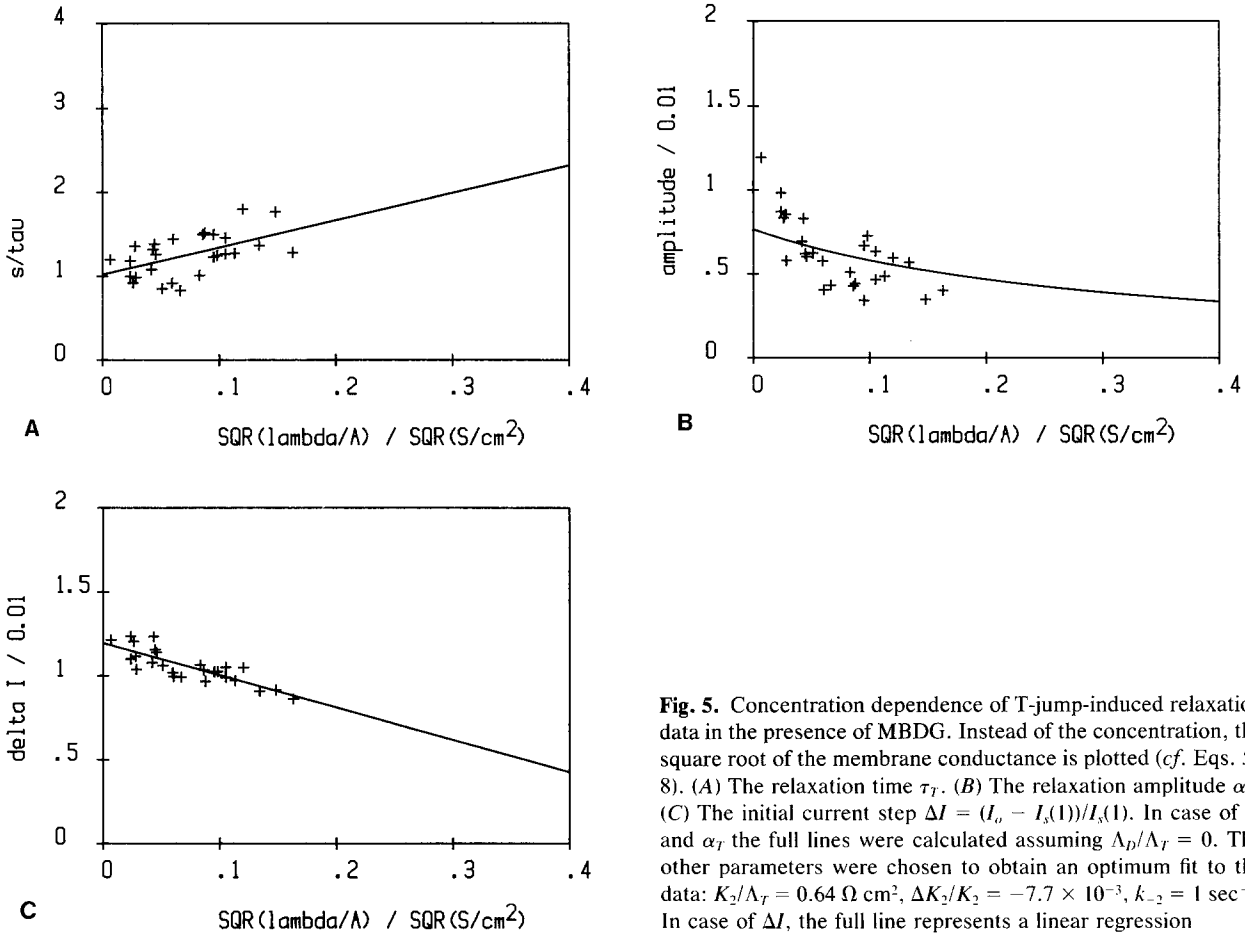


**Fig. 5.** Concentration dependence of T-jump-induced relaxation data in the presence of MBDG. Instead of the concentration, the square root of the membrane conductance is plotted (*cf.* Eqs. 5–8). (*A*) The relaxation time $\tau_T$. (*B*) The relaxation amplitude $\alpha_T$. (*C*) The initial current step $\Delta I = (I_o - I_s(1))/I_s(1)$. In case of $\tau_T$ and $\alpha_T$ the full lines were calculated assuming $\Lambda_D/\Lambda_T = 0$. The other parameters were chosen to obtain an optimum fit to the data: $K_2/\Lambda_T = 0.64\ \Omega\ \text{cm}^2$, $\Delta K_2/K_2 = -7.7 \times 10^{-3}$, $k_{-2} = 1\ \text{sec}^{-1}$. In case of $\Delta I$, the full line represents a linear regression

concentrations, i.e. membrane conductances $\lambda$. The results are shown in Figs. 3*A*–3*C*. In addition to $\tau_T$ and $\alpha_T$ the initial current step, $\Delta J = J_o - J_s(1)$, is plotted as a function of $\sqrt{\lambda}$.

Similar experiments were performed for the negatively charged gramicidin analogue O-pyromellitylgramicidin and for chemically dimerized malonyl-bis-desformylgramicidin. In all these cases the same time course of $J(t)$ was observed. Because of their importance for the following interpretation, the data obtained for MBDG are shown in Figs. 4 and 5.

The relaxation time $\tau_T$ of the T-jump experiments was found (within the experimental error) to be identical to the fast relaxation time $\tau_1$ of the V-jump experiments (*see* Table). The latter was interpreted by Bamberg and Läuger [6] on the basis of a dimerization reaction. According to their model, gramicidin monomers associate to dimers representing the active form of the ion channel. For the chemically dimerized MBDG, Bamberg and Janko [5] reported V-jump-induced current relaxations of considerably smaller amplitude than for GA. This

**Table.** Comparison of the model parameters (*cf.* Eqs. 5–8) for GA, OPG, and MBDG evaluated by T-jump and V-jump experiments[a]

| Analogue | Method | $K_2/\Lambda_T/\Omega\ \text{cm}^2$ | $\Delta K_2/K_2$ | $k_{-2}/\text{sec}^{-1}$ |
|---|---|---|---|---|
| GA | T | 9.6 | −0.011 | 0.7 |
| GA | V | 14.4 | +0.4 | 0.6 |
| OPG | T | — | −0.013 | 1.2 |
| OPG | V | — | +0.23 | 1.9 |
| MBDG | T | (0.6) | −0.008 | 1 |
| MBDG | V | (0.2) | +0.12 | 1.5 |

[a] The T-jump experiments were performed at a constant voltage of 50 mV. The amplitude of the V jump was 80 mV. The data were analyzed assuming $\Lambda_D/\Lambda_T = 0$. The rate constant $k_{-2}$ is identical to $1/\tau$ in the limit $\sqrt{\lambda} \to 0$. Likewise, $\Delta K_2/K_2$ corresponds to $\alpha(\sqrt{\lambda} \to 0)$.

was considered as a further support for the dimeric nature of the active (open) channel. In the T-jump experiments of the present study, however, largely identical behavior of GA and MBDG was found for the relaxation time $\tau_T$ and the relaxation amplitude

$\alpha_T$ (*cf.* Figs. 2–5). Though the amplitude was found to be smaller for MBDG in the V-jump experiments (*see below*), an identical shape of the relaxation curve for both substances was observed, too. These observations have led us to reinterpret the dimerization as an association of two dimers to a tetramer. For a further quantitative interpretation of our data the following model was used: Gramicidin monomers $M$ may form dimers, in line with the previous model. Dimers $D$ may associate to tetramers $T$.

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D$$
$$D + D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} T. \tag{3}$$

In view of the experimental result of an identical behavior of monomeric GA and dimeric MBDG, we assume that the equilibrium of step 1 is strongly in favor of the dimers $D$, i.e. the concentration $N_M$ of monomers $M$ inside the membrane is neglected. We further neglect the exchange of gramicidin between membrane and water. Both dimers $D$ and tetramers $T$ are assumed to form active (open) channels of different conductances $\Lambda_D$ and $\Lambda_T$. Then, the membrane current at an applied voltage $V$ is given by

$$J(t) = (N_D(t)\Lambda_D + N_T(t)\Lambda_T)V. \tag{4}$$

The time-dependence of $N_D$ and $N_T$ is obtained by standard techniques (*see* e.g. [8]). Only step 2 of Eq. (3) is considered (i.e. $N_m \to 0$) and the treatment is restricted to small perturbations of the system ($\Delta K_2 \ll K_2$, $K_2 = k_2/k_{-2}$). The result in form of Eq. (5) agrees with Eq. (1), the function used for the fitting procedure, apart from the correction term of the latter.

$$J(t) = J_s(1 + \alpha \exp(-t/\tau)), \tag{5}$$

$$\frac{1}{\tau} = (4K_2N_D + 1)k_{-2} \tag{6}$$

$$\alpha = \frac{1}{4K_2N_D + 1} \frac{2\Lambda_D/\Lambda_T - 1}{(\Lambda_D/\Lambda_T)/K_2N_D + 1} \frac{\Delta K_2}{K_2} \tag{7}$$

$$K_2N_D = \sqrt{\frac{K_2}{\Lambda_T}\lambda + \left(\frac{\Lambda_D}{2\Lambda_T}\right)^2} - \frac{\Lambda_D}{2\Lambda_T}. \tag{8}$$

Equations (5–8) hold for both, T-jump and V-jump experiments. The magnitude of $\Delta K_2$ can, however, be very different for both kinds of kinetic experiments. Consequently, the amplitude $\alpha$ can be close to zero for a V-jump-induced relaxation while assuming comparatively large values for a T-jump experiment and vice versa. $\alpha$ and $\tau$ are determined by the parameters $K_2/\Lambda_T$, $\Lambda_D/\Lambda_T$, $\Delta K_2/K_2$ and $k_{-2}$, which can be evaluated by a fit of Eqs. (6) and (7) to the experimental data. $K_2/\Lambda_T$ determines the dependence of both $\alpha$ and $\tau$ from the membrane conductance $\lambda$. The fit of both quantities by a common set of parameters is, therefore, a test of the underlying model. This was tried in Figs. 3*A* and *B* and Figs. 5*A* and *B*. The full lines were calculated assuming $\Lambda_D = 0$. In view of the large scatter of the experimental data, an estimate of the ratio $\Lambda_D/\Lambda_T$ is difficult. For $\Lambda_D > 0$, the relaxation amplitude shows a maximum (*see* broken line in Figs. 3*A*, *B*). For $\Lambda_D = \Lambda_T/2$, $\alpha$ is equal to zero, since the association of two dimers to a tetramer is not accompanied by a change of the conductance under these conditions. Though there is no indication of a maximum in the $\alpha$ versus $\sqrt{\lambda}$ relationship, a definite conclusion about the ratio $\Lambda_D/\Lambda_T$ was not obtained because of the large scatter of the data at small values of $\lambda$. If $\Lambda_D/\Lambda_T$ is treated as an additional free variable, Marquardt's procedure of minimizing chi-square [9] in case of GA (Figs. 3*A* and *B*) yields $\Lambda_D/\Lambda_T = 0.04$, $K_2/\Lambda_T = 47.3\ \Omega\ \text{cm}^2$, $\Delta K_2/K_2 = -0.02$ and $k_{-2} = 0.8\ \text{sec}^{-1}$. The value $\Lambda_D \approx 0$ is, therefore, considered as the most probable one.

The test of the applied model would be more rigorous, if the scatter of the experimental data could be reduced. The scatter mainly reflects the heterogeneity of different membranes. If independent experiments are performed at the same membrane, the standard deviation of the values of both quantities, $\alpha$ and $\tau$, from their mean is about 10%. If different membranes are compared at approximately identical $\sqrt{\lambda}$, the standard deviation may be as large as 40% of the mean values. The heterogeneity of the membranes is smaller in the presence of a large voltage ($V \geq 120$ mV). For V-jump experiments the scatter of the data is considerably reduced in this case. A good agreement with the requirements of a dimerization reaction is found [6]. At T-jump experiments a constant voltage is applied over a relatively long period of time. The selection of high-voltage amplitudes is difficult in this case because of the limited membrane stability.

The behavior of dimeric MBDG corresponds to that of monomeric GA. The measurements were restricted, however, to a smaller conductance range, since the maximum achievable conductance was smaller. The decrease of $\alpha$ and $\tau$ with increasing $\sqrt{\lambda}$ is, therefore, less significant. For the analogue OPG (*data not shown*) $\alpha$ and $\tau$ were found to be independent from $\sqrt{\lambda}$ within the experimental error, i.e. $K_2/\Lambda_T \ll 1$ (*cf.* Eqs. 6–8). The results are summarized in the Table. They are compared with corre-

sponding V-jump experiments. For experimental reasons the relaxation of the current can be observed for a considerably larger period of time in this case. The T-jump method is restricted to a few seconds because of the limited temperature stability. Evidence for more than one exponential term has been reported earlier [5, 21, 27] though not investigated in detail. We applied a V jump for 200 sec and we found a continuous increase of the current over this time range which is well described by the function

$$J(t) = J_s(1 - \alpha_1 \exp(-t/\tau_1) - \alpha_2 \exp(-t/\tau_2)) + \beta_V t, \quad (9)$$

$$J_s = J_o/(1 - \alpha_1 - \alpha_2). \quad (10)$$

The same time course was found for normal GA (*not shown*), for OPG (Fig. 6) and for MBDG (Fig. 7). The linear term $\beta_v t$ ($\beta_v > 0$) was introduced to account for a small drift. The latter might be the consequence of a third relaxation process with a considerably larger time constant. So far, only the fast relaxation process ($\alpha_1$, $\tau_1$) had been analyzed and interpreted on the basis of a voltage-dependent monomer-dimer equilibrium [6]. The slower processes as well as the relaxation found in presence of MBDG were considered as side effects not involved in the dimerization process [5]. The following arguments indicate, however, that both phenomena represent important consequences of the mechanism of channel activation.

The time course of the relaxation, apart from small differences in the relaxation times, is identical for monomeric GA and for dimeric MBDG regardless of whether the relaxation is excited by a V or a T jump. Though the amplitudes $\alpha_1$ and $\alpha_2$ of the V-jump-induced relaxation are smaller for MBDG by about a factor 3, there is no reason to assume a fundamental difference in the behavior of the dimeric compound. According to Eq. (7) the amplitude is determined by $\Delta K_2/K_2$ which may be equal or different for both substances. The T-jump amplitude $\alpha_T$ is within the experimental error identical for both substances.

It was argued [4, 5, 21] that the relaxation of the current observed at longer times (seconds to minutes) after a V jump of large amplitude might be influenced by a change of the geometric dimensions of the membrane (membrane area, membrane torus). Consequently, the occurrence of the slower relaxation process was considered as an artifact. Capacitive effects have been reported to depend on the second power of the membrane voltage [1, 54]. Therefore, the voltage dependence of the V-jump-induced relaxation was studied. While previous experiments were mainly performed at relatively large voltage amplitudes ($V \geq 100$ mV), in the present study the amplitude was varied from 40, 80, 120 to 160 mV. At all voltages, the result was similar to that illustrated in Figs. 6 and 7. The voltage dependence of the current amplitudes may be characterized by the total current change $\Delta J/J_o = (J_s - J_o)/J_o$. Using Eq. (10) one finds

$$\Delta J/J_o = (\alpha_1 + \alpha_2)/(1 - \alpha_1 - \alpha_2). \quad (11)$$

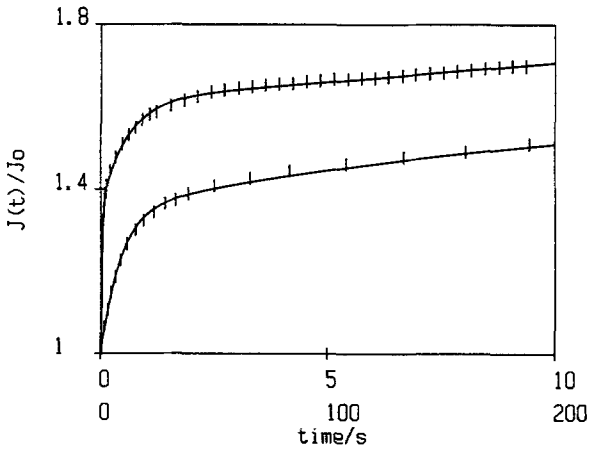


**Fig. 6.** Relaxation of the electric current following a voltage jump of 80 mV in the presence of O-pyromellitylgramicidin. The center of the bars represents the data used for their fitting to Eq. (9). The length of the bars indicates the measurement uncertainty. The initial current density $J_o$ was $1.3 \times 10^{-5}$ A/cm$^2$. The full lines were calculated with the values $\tau_1 = 0.44$ sec, $\tau_2 = 11.2$ sec, $\alpha_1 = 0.22$, $\alpha_2 = 0.17$, $\beta_v = 4.6 \times 10^{-4}$ sec$^{-1}$

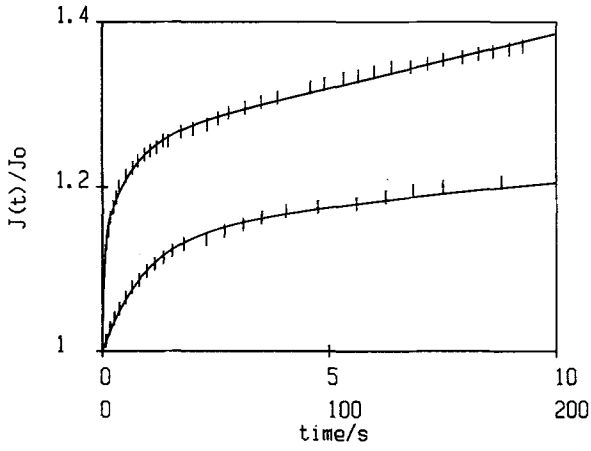


**Fig. 7.** Relaxation of the electric current following a voltage jump of 80 mV in the presence of malonyl-bis-desformylgramicidin. The center of the bars represents the data used for their fitting to Eq. (9). The length of the bars indicates the measurement uncertainty. The initial current density $J_o$ was $2.6 \times 10^{-6}$ A/cm$^2$. The full lines were calculated using $\tau_1 = 0.91$ sec, $\tau_2 = 12.7$ sec, $\alpha_1 = 0.11$, $\alpha_2 = 0.095$, $\beta_v = 6.6 \times 10^{-4}$ sec$^{-1}$

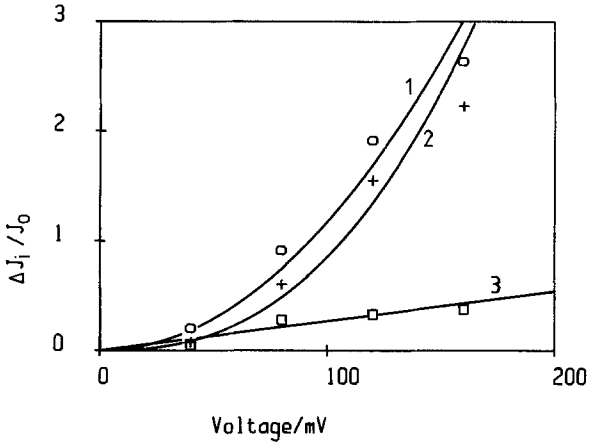


**Fig. 8.** Voltage dependence of the current amplitudes $\Delta J_i/J_o$ according to Eqs. (11) and (12) found at voltage-jump experiments in the presence of gramicidin A. The data refer to zero membrane conductance and were obtained by extrapolation from the dependence of $\alpha_1$ and $\alpha_2$ on the membrane conductance (similar to Figs. 3*B* and 5*B*). The full lines represent a least-square fit of the function $aV^n$ to the data. The parameters $a$ and $n$ were adjusted for optimum fit. Curve 1 (open circles): $\Delta J/J_o = 1.1 \times 10^{-4}$ (voltage/mV)$^{2.02}$; curve 2 (crosses): $\Delta J_1/J_o = 7.8 \times 10^{-6}$ (voltage/mV)$^{2.52}$; curve 3 (open squares): $\Delta J_2/J_o = 2.6 \times 10^{-3}$ (voltage/mV)$^{1.01}$

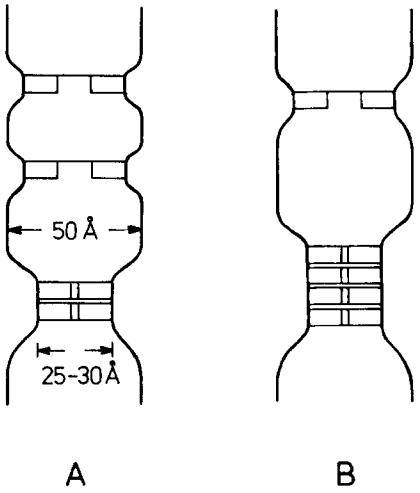


**Fig. 9.** Schematic illustration of the proposed model for the aggregation behavior of gramicidin dimers. (*A*) Nonconducting dimers are transferred into the conducting state by association to tetramers. (*B*) Tetramers represent nuclei for the formation of larger aggregates of conducting gramicidin dimers

$\Delta J/J_o$ is the sum of the two relaxation components

$$\Delta J_1/J_o = \alpha_1/(1 - \alpha_1 - \alpha_2)$$

and (12)

$$\Delta J_2/J_o = \alpha_2/(1 - \alpha_1 - \alpha_2).$$

The following approximate voltage dependences were found (*see* Fig. 8):

$$\begin{aligned} &\Delta J/J_o \sim V^k,\ k \approx 2 \\ &\Delta J_1/J_o \sim V^l,\ l \approx 2.5 \\ &\Delta J_2/J_o \sim V^m,\ m \approx 1. \end{aligned} \quad (13)$$

The component $\Delta J_2$ increases linearly with the voltage whereas a second power dependence was reported for capacitive effects. The ratio $\Delta J_2/\Delta J_1$ (i.e. the relative importance of $\Delta J_2$) decreases with increasing voltage ($\Delta J_2/\Delta J_1 \sim V^{-1.5}$). At 40 mV, $\Delta J_2$ was found to be comparable to $\Delta J_1$. If $\Delta J_2$ were the consequence of a voltage effect on the membrane area, rather an opposite behavior, namely an increasing importance at high voltages, would be expected. Therefore, we believe that relaxation process 2 mirrors an intrinsic property of the gramicidin system. In agreement with previous studies we believe that the same is true to the fast relaxation process 1, since there is independent evidence of this process from noise analysis [27–29, 55] and from the T-jump method applied in our present study. Both methods work at constant membrane voltage.

If the slow relaxation process is treated as a "true" relaxation of the gramicidin system, our model of channel formation leading to Eqs. (5–8) must be extended. The concentration dependence of the fast relaxation process was found to agree with the T-jump data within the experimental accuracy (*cf.* Table). The latter were interpreted on the basis of an association of nonconducting dimers to conducting tetramers. We suggest that the slow relaxation process (including the long-lasting drift) indicates a further association of dimers to pre-existing tetramers. In this way larger aggregates of channels are formed. The model is illustrated in Fig. 9. It is based on a parallel association of the dimers. A serial arrangement of two dimers to a tetramer (and a subsequent parallel association of tetramers) can be excluded considering the results obtained with OPG. This gramicidin analogue bears three negative charges at the C-terminal end and behaves largely identical to normal GA with respect to the kinetic properties investigated in the present study. A serial association of two OPG-dimers requires the transfer of six charges into the hydrophobic membrane interior. It is, therefore, highly improbable for energetic reasons.

The application of a V jump represents a driving force towards the formation of new aggregates or the enlargement of small ones. The association of two nonconducting dimers to a conducting tetramer

is the first step in this process. It must be left open whether there is a direct influence of the electric field on GA, favoring channel aggregation, as was suggested by Urry [47], or whether aggregation is induced indirectly via a field effect on the membrane structure. Bamberg and Benz [4] found that the time course of the relaxation observed in the presence of GA did not correlate with voltage-induced thickness changes of the membrane measured via the membrane capacitance. Therefore a direct field effect on the helical structure of the channel was supposed. Fröhlich [16] reported a strong influence on the voltage-jump-induced relaxation by structural properties of the membrane. For asymmetric bilayers the presence of a membrane-internal asymmetry potential was postulated. We would like to draw the attention to the possibility of a field effect on the membrane structure which does not necessarily result in measurable capacitive changes of the membrane and which might be indirectly responsible for the V-jump-induced GA relaxations. It is well known that planar lipid membranes are inhomogeneous with respect to the membrane thickness. Apart from the intrinsic heterogeneity, there is an uneven distribution of solvent leading to the appearance of microlenses [38]. The spatial variation of the membrane thickness may give rise to nonideal current relaxations found in the presence of hydrophobic ions [25]. The compression of the membrane in the presence of a voltage is strongest at the thinnest domains of the membrane. Consequently, thinner domains will grow at the expense of thicker ones. The increase of the capacitance associated with this process could be largely compensated for through an increasing thickness of the thicker domains. The length of a GA dimer of the Urry-type is about 25 to 30 Å, i.e. considerably shorter than the thickness of a typical bilayer membrane. The bridging of the membrane by a GA dimer will only be possible at the thinnest domains. Even there, the insertion of a dimer requires an additional local thinning of the membrane [6, 23, 24, 28]. This process is energetically not favorable and will exert considerable mechanical stress to the two helices forming the dimer. The consequence of the resulting force might be a partial breaking of the intermolecular hydrogen bonds between the two monomers leading to a substantial increase of the inner barrier which is responsible for the rate of ion movement through the channel. In this way the conductance of the isolated dimeric channel could be strongly reduced. The association of two dimers to a tetramer, even more so the formation of larger aggregates, is suggested to improve the energetic situation of the single dimers, leading to a channel conformation with a reduced inner barrier, i.e. giving rise to a strong increase of the conductance. Thus the V-jump experiments may be explained as a voltage-induced increase of the area occupied by the domains of comparatively small membrane thickness. Thereby, channel aggregation is favored. The T-jump experiments on the other hand are interpreted as a decrease of the number of aggregated dimers with increasing temperature.

## Discussion

The aggregational model of the gramicidin A ion channel illustrated in Fig. 9 is essentially based on two experimental findings, namely a nearly identical relaxation behavior of monomer gramicidin A and chemically dimerized malonyl-bis-desformyl-gramicidin in the T-jump experiments and the establishment of a second relaxation time in the range of seconds as a "true" relaxation process of the gramicidin system. Both findings are incompatible with the single monomer-dimer equilibrium used so far to explain the formation and the decay of gramicidin channels.

Previous experiments with the dimerized compound MBDG [5, 40, 48] have been interpreted as a support for the monomer-dimer hypothesis. A critical inspection of these studies shows, however, that the interpretation given by the authors is questionable and cannot be used as an argument in favor of the monomer-dimer hypothesis. Urry et al. [48] reported a linear variation of the membrane conductance versus the concentration of MBDG in water, while normal GA shows a quadratic dependence. In view of the very poor water solubility of GA and MBDG, the results of such experiments must be regarded as very doubtful. The molecular state of GA and MBDG in water is not known. Aggregation phenomena cannot be excluded. The presence of aggregates in water is, however, of great importance, if the molecularity of the aggregation reaction in the membrane is deduced from the concentration dependence of the conductance (*see* discussion below and also Appendix B). Because of the unclear state of GA and MBDG in water other authors [6, 28, 52, 55] have avoided specifying the concentrations of these compounds in water. Instead, the conductance was used as an indirect means to characterize the amount of GA in the membrane. There is another reason why the different behavior of GA and MBDG reported by Urry et al. [48] appears unclear. Veatch found that most of the dimeric compound of these authors was monomer, i.e. the covalent dimer was a minor component [49]. Bamberg and Janko [5] and Sauvé and Bamberg [40] used a carefully purified sample of MBDG

and found characteristic differences in the behavior of this compound compared to monomer GA. The life-time of MBDG single channels was about two orders of magnitude larger than those of monomer GA. In addition a characteristic flickering was observed [5]. The amplitude of the current relaxation following a voltage jump was considerably smaller for MBDG. The power spectrum density $S(f)$ of the electric current showed a $1/f$ behavior in case of MBDG [40], while that of normal GA is of the Lorentz-type ($S(f) \sim 1/f^2$, $f > f_c$, $f_c$ = corner frequency) [28, 55]. The differences between GA and MBDG observed in the relaxation experiments and in the noise behavior were interpreted by the authors as an evidence for the absence of a dimerization step in case of MBDG. Though we have repeated and confirmed the essential experimental results obtained by Bamberg and Janko [5] and by Sauvé and Bamberg [40], we would like to present the following objections against their interpretation. The reduction of the amplitude of a relaxation process can be used as an argument against the existence of the underlying molecular process (namely the dimerization) only, if the amplitude is zero. This is certainly not the case, as is clearly apparent from Fig. 3 of reference [5] and from the more detailed analysis of the shape of the relaxation curves performed during the present study (*compare* Figs. 6 and 7). The argument against the dimerization of MBDG based on the shape of the power spectrum has recently been disproved by one of the authors of reference [40]. It was found that the power spectrum induced by MBDG can be explained by a sum of several Lorentzians [41] (*see also* ref. [26]). This means that the electrical noise observed in the presence of MBDG may be explained as a superposition of several types of opening-closing kinetics, i.e. the fluctuation behavior of MBDG (though more complicated) seems to resemble that of normal GA.

There is a series of further experimental approaches which is often cited in support of the monomer-dimer hypothesis. One of these is the concentration dependence of the conductance of the gramicidin analogue OPG. The latter bears three negative charges at its C-terminal end providing for a better water solubility. Apell et al. [2] found a second-order dependence of the conductance versus concentration in water extending over two orders of magnitude of the concentration. A further important finding of the authors was that OPG must be added to the aqueous solutions on both sides of the membrane in order to obtain active ion channels. Therefore, it has to be concluded that the aggregate responsible for an open channel is formed by association of monomers coming from opposite aqueous phases. The conclusion that the open channel is formed by a dimer is, however, premature. Such an inference, derived from the second-order concentration dependence of the conductance [2], is only correct, if the molecular state of OPG in water is monomeric. The latter has not been studied so far. Veatch et al. [49, 50] investigated the aggregation behavior of gramicidin A in organic solvents of different polarity by circular dichroism, fluorescence measurements and quantitative thin-layer chromatography. Clear evidence for a dimerization reaction was found. Similar studies in water are difficult since they must be performed in a concentration range far below that used for the organic solvents. Though experimental data for water are not available, the existence of dimers and larger aggregates cannot be excluded. If it is assumed that OPG in water mainly exists in the form of dimers in the considered concentration range, and if the exchange of molecules between membrane and water proceeds via the monomers, then a tetramer structure of the open channel has to be inferred from the experimental finding of a second-order concentration dependence of the conductance (*see* Appendix B). In consequence, the aggregational behavior in water and the mechanism of exchange between water and membrane must be known, before a reliable prediction can be derived from the conductance-concentration relationship. This is, at least at present, not possible in the case of gramicidin A and its analogues.

Further support for the monomer-dimer hypothesis was published by Veatch et al. [52]. Simultaneous fluorescence and conductance studies of a fluorescent analogue of gramicidin A in planar bilayer membranes were performed. The relationship between the conductance (which is proportional to the total concentration of open channels) and the fluorescence (proportional to the total membrane concentration of the gramicidin analogue) was found to be in fair agreement with the predictions of a dimerization reaction. A discrimination between the dimer and the tetramer hypothesis is not possible, however. If the monomer-dimer equilibrium of Eq. (3) is strongly in favor of the dimers, only the second step of the reaction scheme, namely the dimerization of dimers to tetramers, will be observed during such an experiment. Veatch et al. [52] further compared the membrane concentration of dansyl-gramicidin C, determined from their fluorescence measurements, with that obtained from the membrane conductance. This was done under conditions, where every gramicidin molecule was supposed to contribute to the membrane conductance. A mean ratio of the concentration values, obtained

by the two methods, of 1,4 was found. Since the experimental error was reported to be a factor of two, this result again cannot be used to discriminate between the dimer-hypothesis (expected ratio 1) and the tetramer hypothesis (expected ratio 2).

Finally the dimer hypothesis was tested by the study of hybrid channels. The latter are observed if single-channel experiments are performed in the presence of two gramicidin analogues which have different values of the single-channel conductance. A third conductance level in between those of the single components was found and was interpreted as a channel formed by both gramicidin analogues [2, 32, 53]. The experimental finding of three conductance levels seems to support the dimer hypothesis since the tetramer hypothesis predicts at least five different conductance levels. If the two different gramicidin analogues are denoted by A and B, the species AAAA, AAAB, AABB, ABBB and BBBB may be expected. In addition, species made up by the same numbers of monomers A and B such as AABB and ABAB might be different with respect to their single-channel properties, thus even enhancing the number of possible conductance levels. The experimental analysis of the number of different conductance states is, however, complicated by the relatively broad distribution of conductance steps found in the presence of only one gramicidin analogue. As a result, two conductance levels can be distinguished from one another only if they are sufficiently separated. If the difference between two species which differ only in a single subunit (such as species AAAA and AAAB or species ABBB and BBBB) is too small, the system degenerates to the three conductance levels originating from the species AAAA, AABB and BBBB. This means that because of the limited resolution of different conductance states both dimer and tetramer hypotheses exhibit the same number of experimentally observable conductance states. Similar arguments apply to the fluorescence energy transfer studies of hybrid channels performed by Veatch and Stryer [53]. Though the authors favor the dimer hypothesis they admit that more complex models (such as the tetramer model) that depend on special assumptions cannot be excluded.

Summarizing, the different experimental approaches used so far to study the quarternary structure of the gramicidin A ion channel are not sufficient to distinguish between the simple dimer hypothesis and more complex models. We have shown that the relaxation behavior of gramicidin A and its analogues in planar lipid membranes is incompatible with the simple dimer model. The aggregational model depicted in Fig. 9 can explain all relevant experimental data. Nevertheless, it certainly is still a "minimum model," i.e. a greatly simplified picture of more complex phenomena.

We will now use this model to discuss our data in the frame of previous results obtained by different methods. A supramolecular organization of gramicidin channels was first postulated by Urry [47] on the basis of his conformational studies. Meanwhile, different experimental approaches provided evidence for the existence of channel aggregates. Cavatorta et al. [14] reported substantial quenching of the tryptophan fluorescence of GA incorporated into lysophosphatidylcholine phospholipid structures. They suggested a network of channels in which the interchannel contacts are established by Trp-Trp interactions. Dielectric relaxation studies at the same system were also interpreted on the basis of parallel-oriented neighboring channels [20]. Finally electron-microsopic investigations of GA in phospholipid micelles demonstrated the presence of a supramolecular organization of particles which were interpreted as hexamers of channels [42]. In the same study the presence of GA was found to convert the micelles into a vesicle-like bilayer structure. In planar (black) lipid membranes, the opening and closing of gramicidin pores was found to occur independently from one another, if measured by the single-channel technique at extremely small concentrations [23]. Noise experiments performed at comparatively large concentrations of GA in long-chain monoglyceride membranes gave, however, considerably lower values of the single-channel conductance, if compared with the values obtained by the single-channel technique at small concentrations [27, 28]. This was explained on the basis of electrostatic interactions between channels arranged in local clusters.

Thus, the model of the gramicidin channel proposed in the present paper is in line with previous findings. Our model provides, however, an extension of previous interpretations, as we assume that isolated gramicidin dimers contribute considerably less (zero conductance cannot be excluded) to the conductance than dimers arranged in tetramers or larger aggregates.

Our data will now be compared with previous relaxation and single-channel experiments.

The initial current step found after application of a T jump (*cf.* Figs. 3*C* and 5*C*) is interpreted as an instantaneous increase of the ion mobility inside the gramicidin channels by the temperature. The amplitude of the applied T jump was 0.25 K. The size $\Delta I = (I_o - I_s(1)/I_s(1))$ of the initial current step was about 0.01. The activation energy of the single-

channel conductance calculated from these values is 28 kJ/mol and compares well with 30.5 kJ/mol obtained by Bamberg and Läuger [7] from single-channel measurements. There is a small but significant decrease of $\Delta I$ with increasing conductance. This might represent an effect of channel aggregation on the single channel conductance. This interpretation is in line with findings from noise experiments of Kolb and Bamberg [28] who reported a reduced single-channel conductance at elevated conductance levels. The enthalpy of tetramer formation $\Delta H$ may be calculated from the relation $\Delta H/RT \approx (\Delta K_2/K_2)(T/\Delta T)$. Assuming $\Delta K_2/K_2 = -0.011$ (*see* Table) one obtains $\Delta H \approx -32$ kJ/mol. This is contrary to the small positive value of $\Delta H$ reported from the temperature dependence of previous V-jump experiments [7]. The previous procedure is subject to considerable error because of the uncertainty in the determination of the individual rate constants.

The rate constant $k_{-2}$ obtained for GA and OPG (*see* Table) agrees with previous V-jump data [2, 6] and with values derived from noise analysis [29, 55] within a factor 2 to 3. The same is true in case of GA to the ratio $K_2/\Lambda_T$. Apell et al. [2] reported 5 times smaller values for OPG than for GA, while our data showed no experimentally significant concentration dependence for OPG, i.e. $K_2/\Lambda_T$ could not be determined in this case. The differences may at least in part be due to the fact that previous data were fitted by a single exponential function, while our data were analyzed by a sum of two exponentials plus a linear term (Eq. (9)). Smaller values of the equilibrium constant $K_2$ may be expected, since the presence of negative charges at OPG disfavors aggregation, if compared with the neutral GA.

Further comparison can be performed with single-channel experiments. The values of the rate constant $k_{-2}$ found for GA (0.7 $sec^{-1}$ and 0.6 $sec^{-1}$) are in reasonable agreement with that derived from the life-time of the single channels (0.9 $sec^{-1}$) [29]. There is, however, a fundamental difference in the previous interpretation of single-channel fluctuations and that suggested in the present paper. For GA as well as for OPG, most experiments provided evidence only for two states of a gramicidin channel (open and closed) [2, 23, 32]. The two states are currently believed to represent (nonconducting) monomers and (conducting) dimers of gramicidin. The nearly identical behavior of GA and MBDG in the T-jump experiments let us, however, doubt this interpretation and suggest tetramers as well as higher gramicidin-aggregates to be mainly responsible for the conductance induced by GA and its analogues. Consequently single-channel fluctuations are believed to reflect the dissociation of tetramers into dimers. A definite conclusion about the single-channel conductance $\Lambda_D$ of the dimer was not obtained, though (as has been discussed in detail above) $\Lambda_D \ll \Lambda_T$ is highly probable. The detection of low-level conductance states in the presence of high-level states is difficult for technical reasons. It is interesting to note in this context that Busath and Szabo [13] observed miniature conductance levels. Their study indicates a far greater complexity of the gramicidin channel than assumed so far. This is also true for dimeric MBDG. Bamberg and Janko [5] reported a rather complex fluctuation behavior of this channel. Though a detailed quantitative analysis was not performed, the authors reported experimental findings which differ from normal GA, namely the observation of channel life-times (in the open state) of several minutes and the existence of very fast transitions (flickering). There is no correspondence between the channel life-times and the relaxation times observed at the T-jump and V-jump experiments in the presence of MBDG. An agreement between channel life-times and chemical relaxation times in the limit of small channel concentrations would be expected, if channel activation proceeds via a bimolecular reaction, and if this process is temperature and voltage dependent. While this agreement is given for normal GA (*see above*), it seems to fail in the presence of MBDG. Our own preliminary single-channel experiments have shown, however, that the single-channel behavior of MBDG is still more complex than described by Bamberg and Janko [5] and needs further investigation. We found that there are channel life-times in the region of seconds (in fair agreement with the reported relaxation times) in addition to the very long-living channels reported previously [5]. The relative frequency of these channels, which have the same amplitude as the long-living channels, is the subject of further study. Similar observations have recently been mentioned by Sauvé and Szabo [41]. These findings are supported by a new interpretation of the noise experiments of Sauvé and Bamberg [40]. The $1/f$-power spectrum density found in the presence of MBDG may be explained by a sum of at least three Lorentzians, characterizing at least three different types of opening-closing events [41]. A detailed analysis has not been published yet. A simple estimate shows, however, that the characteristic time constants of the Lorentzians have to cover a broad time range from milliseconds to at least seconds in order to fit the experimental data. This is clearly at variance with the single-channel data of Bamberg and Janko [5], which show only two kinds of opening-closing events. It is, however, in line with our own findings and those of Sauvé and Szabo [41].

The different time constants supplied by the various methods as well as the observation of miniature conductance levels [13] indicate that the reaction scheme proposed in the present paper is still incomplete. It will have to be supplemented by additional "channel states" in the future. We think, however, that our model represents a basis for more detailed investigations.

There is a further important consequence of our model for the interpretation of single-channel conductance data. If both dimers of a tetramer unit are in the open state, the values assumed so far for the conductance of a single dimer have to be corrected by a factor of two. No correction is necessary, if the association of two dimers to a tetramer leads to the opening of only one of the two dimeric channels. Then, however, the two dimers of a tetramer would be nonequivalent. Though there is no definite evidence for one of the two alternatives, the assumption of a concerted opening of the two dimers allows to explain a discrepancy found during autocorrelation studies [28]. The data obtained from these studies show a reduction of the single-unit conductance at large membrane conductance. Such a behavior is in line with an aggregational model. At small membrane conductance, current fluctuations are mainly due to the formation and dissociation of tetramers, i.e. they correspond to the contribution of two dimers. At large membrane conductance, the fluctuations arise mainly from the association and dissociation of single dimers to larger channel aggregates; i.e. the fluctuations correspond to the contribution of single dimers.

We are still far away from a complete understanding of the molecular structure of the gramicidin A ion channel. An improved analysis of single-channel studies in combination with refined relaxation experiments including noise analysis and the use of further chemical analogues will provide a better insight into the molecular details of an ion channel, which is currently thought to represent the simplest model for ion channels in biological membranes. The dimer hypothesis of the gramicidin channel has been an exception under the molecular models for channel formers of comparatively low molecular weight. For compounds such as alamethicin [10, 17], monazomycin [34] as well as for the polyenes nystatin and amphotericin [15] micelle-like aggregates of rather high molecularity have been proposed. We suggest an aggregational model also for the gramicidin A ion channel.

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## Appendix A: Correction of the T-Jump-Induced Relaxation for the Series Resistance of the Aqueous Phase

In order to avoid photoartifacts at the electrodes, these were light shielded and placed in separate vessels connected to the bilayer cuvette by salt bridges [12]. Thereby, the series resistance $R_s$ to the membrane was increased (1 kΩ at 1 M NaCl solutions). At low membrane conductance, $R_s$ could be neglected. At high conductance, the current was corrected in the following way:

The observed current $J(t)$ after a T jump is

$$J(t) = V/(R_m(t) + R_s). \tag{A1}$$

$V$ = applied voltage, $R_m(t)$ = time-dependent membrane resistance.

The current $J_{co}(t)$ corrected for the voltage drop at $R_s$ is

$$J_{co}(t) = V/R_m(t). \tag{A2}$$

Combining Eqs. (A1) and (A2) one obtains

$$J_{co}(t) = J(t)/(1 - J(t)R_s/V). \tag{A3}$$

The correction assumes that $R_m(t)$ is not influenced by the voltage drop at $R_s$. A simple estimate based on the results of the V-jump experiments shows that this approximation is very well fulfilled.

Equation (A3) was used to correct the measured current data. The corrected data were then fitted by Eq. (1). The correction affects only the relaxation amplitude and the initial current step, while the relaxation time is not influenced. This is shown for time and amplitude as follows: Suppose, the membrane conductance $\lambda_m = 1/R_m$ shows a simple exponential relaxation

$$\lambda_m(t) = \lambda_m^s(1 + \alpha \exp(-t/\tau)), \tag{A4}$$

with $\alpha \ll 1$.

Then, using Eqs. (A1) and (A4), the reduced current $F(t) = J(t)/J_s$ in the limit $\alpha \ll 1$ is calculated as

$$F(t) = 1 + \frac{\alpha}{1 + c} \exp(-t/\tau), \tag{A5}$$

$c = R_s/R_m^s$, $R_m^s$ = steady-state value of the membrane conductance. The presence of $R_s$ is accounted for through the amplitude correction factor $(1 + c)$.

## Appendix B: Concentration Dependence of the Membrane Conductance

The membrane conductance was reported to depend on the second power of the concentration of negatively charged OPG [2]. The following model shows how this experimental finding can be explained on the basis of the tetramer hypothesis.

We assume that OPG can form dimers in the membrane as well as in the aqueous phase. The equilibrium in water is characterized through

$$C_D = K_w C_M^2. \tag{B1}$$

$C_D, C_M$ = concentrations of dimers and monomers in water. The total concentration $C_t$ of OPG monomers in water is

$$C_t = C_M + 2C_D. \tag{B2}$$

From Eqs. (B1) and (B2)

$$C_M = -\frac{1}{4K_w} + \sqrt{\frac{C_t}{2K_w} + \frac{1}{16K_w^2}}. \tag{B3}$$

If the equilibrium in water is in favor of the dimers within the considered range of concentration, i.e. $C_t \gg \frac{1}{8}K_w$, Eq. (B3) is reduced to

$$C_M = \sqrt{\frac{C_t}{2K_w}}. \tag{B4}$$

We further assume that the interfacial concentration $N_m$ of monomers in the membrane is related to the monomer concentration in water by a simple partition equilibrium

$$N_M = \beta C_M, \tag{B5}$$

where $\beta$ = partition coefficient.

According to the proposed reaction scheme (Eq. 3), monomers from opposite interfaces may combine to dimers $N_D$ and dimers may associate to tetramers $N_T$. Therefore, $N_T = K_1 K_2 N_M^4$ and using Eqs. (B4) and (B5)

$$N_T = \frac{K_1 K_2 \beta^4}{4K_w^2} C_t^2. \tag{B6}$$

According to the tetramer hypothesis, only tetramers (as well as larger aggregates neglected in the present simplified treatment) form conducting channels. Then, the membrane conductance $\lambda$ is proportional to $N_T$ and depends on the second power of the total OPG concentration $C_t$ in water.

The study of the aggregation behavior of OPG in water would represent a critical test of the assumptions used to derive Eq. (B6).