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THE ACTION OF A CARBONSUBOXIDE DIMERIZED GRAMICIDIN A ON LIPID BILAYER MEMBRANES

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Summary

Gramicidin A was dimerized with carbonsuboxide as bifunctional reagent. The effect of the resulting malonyl-bis-desformylgramicidin on lipid bilayer membranes was investigated and compared with the effect of the monomer gramicidin. It was found that the single channel conductance and the ion selectivity are very similar to the behaviour of the monomer molecule, whereas the channel forming kinetics and the life time of the single channel of the malonyl-bis-desformylgramicidin differ strongly from the behaviour of the monomer gramicidin.

The electrical relaxations are very small and possibly associated with some structural changes of the membrane after a voltage jump. The single channel lifetime of the malonyl-bis-desformylgramicidin is measured in minutes, whereas for the same lipid system the single channel lifetime in the case of the monomer gramicidin is restricted to 1—2 s. It is concluded that the malonyl-bis-desformylgramicidin-molecule itself (as a single molecule) forms an ionic channel without further association.

Introduction

Gramicidin A, a linear hydrophobic pentadecapeptide isolated from *Bacillus brevis* induces cation selective channels on lipid bilayer membranes [1—14]. According to a proposal by Urry [15—17] the structure of such a gramicidin channel consists of a helix which is formed by head-to-head association of two gramicidin A molecules in the membrane. This hypothesis is supported by the findings that the chemically dimerized malonyl-bis-desformylgramicidin increases the membrane conductance [7,11] and creates single channels in the membrane. According to Urry, the gramicidin channel is a $\pi_{L,D}$ helix. The diameter of the hydrophilic interior of this helix is 4 Å. Recently Veatch and Blout proposed an alternate structure for the gramicidin channel, in which two

gramicidin molecules consist of a double helix [18].

The dimerization process in the membrane was investigated with electrical relaxation experiments [12]. These results are confirmed by experiments of Zingsheim and Neher [19] and Kolb et al. [20]. These authors analyzed the electrical noise induced by the opening and closing of the gramicidin channels in a multichannel membrane system and found similar results as were found with the relaxation method. Recently it was shown by Bamberg and Benz that an electrical field can directly influence the dimerization process of gramicidin in the membrane [14]. Veatch et al. demonstrated with the aid of fluorescent labelled gramicidin C that the channel formation follows a second order reaction. They measured simultaneously the fluorescence of gramicidin in the membrane and the conductance of the bilayer [21].

The aim of this paper is to study the kinetic parameters of a chemically dimerized gramicidin and to compare these results with those obtained from experiments with the monomer gramicidin.

Materials and Methods

(a) For the dimerization synthesis a commercially available gramicidin was used (Nutritional Biochemical Corporation, Cleveland, Ohio). This product contains 72% gramicidin A, 9% gramicidin B and 19% gramicidin C [22]. The preparative purification of malonyl-bis-desformylgramicidin was carried out on a calibrated Sephadex LH 20 gel column. The length of the column was 2 m and the radius about 2 cm. The column had 10 000 theoretical plates.

The ultraviolet spectral data were obtained with a Zeiss DMR 10 spectrophotometer, whereas the infrared spectra were performed with a Perkin Elmer IR spectrometer 577.

The NMR runs were carried out with a 380 MHz Bruker spectrometer. The molecular weight of malonyl-bis-gramicidin was determined by Sephadex LH 20 permeation chromatography and in an analytical ultracentrifuge (Beckman E-IM-3).

(b) Optically black lipid membranes were formed in the usual way from a 1—2% (w/v) lipid solution in *n*-decane in a thermostated Teflon cell filled with electrolyte solution [23]. The area of the membranes was $8 \cdot 10^{-2}$ cm^2 for voltage jump experiments and $3 \cdot 10^{-2}$ cm^2 for measurements of biionic potentials. For measurements of the single channel conductance an area of about 10^{-3} cm^2 was used. In the case of the relaxation experiments the torus was kept smaller than 5—10% of the total area. Dioleoyl phosphatidylcholine, diphytanoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine were synthetized and chromatographically purified in our laboratory.

The purified gramicidin A for the electrical measurements was a gift of Dr. E. Gross, Bethesda, Md. Small amounts of a methanolic stock solution were added to the electrolyte solution. Malonyl-bis-desformylgramicidin was added in small amounts (5—10 μl) to the electrolyte from a methanolic stock solution (10^{-4}—10^{-6} M). Occasionally the peptide was added to the lipid phase and to the electrolyte. Both types of experiments gave the same result.

The relaxation experiments were carried out as described previously [12] by applying a voltage (50—200 mV) to the membrane through silver-silver chloride

electrodes by means of a fast electronic switch (rise time about 1 μs). The time course of the current was recorded with a Tektronix 51003 storage oscilloscope as a voltage drop across an external resistor. The rise time of the voltage which is rendered by the product of the membrane capacity and the resistor of the external circuit, was in the range of 1—100 μs. For measurements of current fluctuations arising from the opening and closing of the single channels [9], the same setup as described previously was employed [13]. The fluctuating current was recorded by a tape recorder (Ampex PR 2200) and, when necessary, the signal was transferred on an expanded time scale to a strip chart recorder.

The dilution and biionic potentials were measured in the following way. At first the solutions on both sides of the membrane were symmetrical. The potential under these conditions was ±1 mV. Then by means of two syringes the electrolyte in one compartment was exchanged. The total volume exchanged was twice the volume of the compartment, so that the differing solutions in the compartment were not mixed during the exchange.

Results and Discussion

(I) Preparation of the malonyl-bis-desformylgramicidin

The chemical dimerization was performed in the following way. Monomer gramicidin was desformylated with methanolic hydrogen chloride according to Shin-ichi Ishii and Witkop [24]. The product was purified of unreacted gramicidin by column chromatography (ionic exchanger). As bifunctional reagent for the dimerization we used carbin suboxide C_3O_2. Carbon suboxide, the double anhydride of malonic acid, is extremely reactive. It is a "clean" reagent which cannot produce active side products such as HCl or chlorinated phenols, unlike the reactions of malonyl chloride or reactive malonic acid esters. Unfortunately, very few publications exist concerning the reactions of C_3O_2 in the presence of amino acids [25] and proteins [26]. Recently it was shown by Sorochinskaya et al. [27], that under certain conditions the carbonsuboxide reacts selectively with the amino residue of amino alcohols as a bifunctional reagent. Since the desformylated gramicidin looks very "similar" to an amino alcohol, the OH group and the amino group being on opposite ends of the molecule, it seems reasonable to use carbonsuboxide as a reagent for the dimerization.

Carbonsuboxide was obtained by pyrogenic destructions of malonic acid, according to Borrmann [28]

$$HOOC - CH_2 - COOH \xrightarrow{P_2O_5} O = C = C = C = O. \quad (1)$$

The resulting product was very pure and was kept in dry ether at −20° C. The activity of the product was tested by the method reported by Staudinger and Bereza [29].

After this procedure a solution of carbonsuboxide in ether was gradually added to a dimethylformamide solution of desformylated gramicidin at −20° C. The final ratio of carbonsuboxide to desformylgramicidin was 2 : 1 (w/w equivalent). The mixture was stored in a refrigerator overnight at 0° C and then evaporated. The reaction is shown in Fig. 1.

In order to verify that the desired dimerized molecule had been obtained,

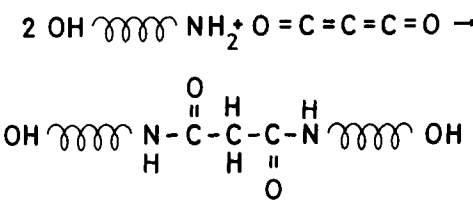

Fig. 1. Schematic representation of the chemical dimerization of gramicidin A.

the product was tested by various methods: (1) by thin-layer chromatography; (2) by LH 20-Sephadex gel permeation chromatography; (3) by different spectroscopic methods (ultraviolet, infrared and NMR); (4) by determination of the molecular weight in an analytical ultracentrifuge.

The thin-layer chromatography revealed the presence of the new product as well as some unreacted monomer desformylgramicidin. The solvent in this procedure was chloroform/methanol/water (65 : 25 : 4, v/v/v). In order to test the new product the malonyl-bis-desformyl gramicidin was removed from the thin-layer plate and measured the electrical properties on lipid membranes. The same results were obtained as for the further purified product, as can be seen in the second part of this paper.

Malonyl-bis-desformylgramicidin was purified by LH 20 Sephadex gel permeation chromatography. Fig. 2 shows the elution diagram. This figure shows that the reaction was not quantitative. An evaluation of the elution diagram gives a yield of 50%. Furthermore, malonyl-bis-desformylgramicidin shows 3 fractions in the elution diagram, whereas the monomer desformylgramicidin produces only one peak. The reason for this is possibly that the gramicidin exists as a mixture of the gramicidins A, B and C which leads to separable dimer products: (A-A, B-B, C-C, A-B, A-C, B-C), whereas the monomer molecules, because of the permeation velocity of the column, do not show this behaviour. However, when the permeation velocity is adjusted to a slower rate, the monomer gramicidin shows the same kind of elution diagram as can be seen in the case of malonyl-bis-desformylgramicidin.

The three fractions of malonyl-bis-desformylgramicidin were examined by infrared-spectroscopy and in single channel experiments on lipid membranes.

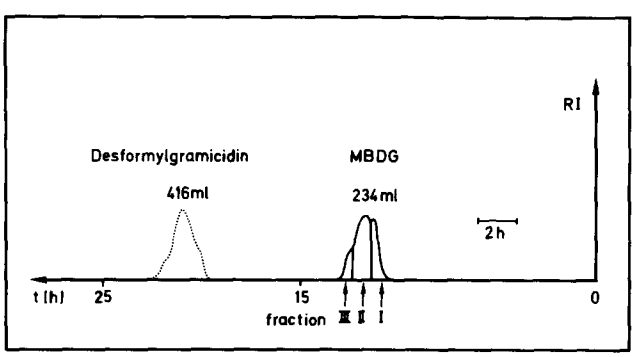

Fig. 2. Elution diagram of the LH-20 Sephadex column. Methanol was used as solvent. RI is the refraction index. I, II, III on the malonyl-bis-desformylgramicidin peak represent the different fractions. MBDG, malonyl-bis-desformylgramicidin.

No differences were detected between the various fractions.

Furthermore, it is possible to determine by Sephadex LH 20 permeation chromatography the molecular weight of the chromatographed substances. In the case of desformylgramicidin a value of approx. 2000 g/mol was found and for the dimerized molecule approx. 4000 g/mol. Previous to this procedure, the column was calibrated with peptides of known molecular weight (valinomycin and some analogues).

In order to show that the gramicidin molecule was not changed with respect to its intrinsic properties other than of the dimerization, several spectroscopic tests with the new molecule were made.

(1) The ultraviolet-spectrum is identical to that of the monomer product, i.e. the tryptophane residues are not damaged.

(2) In the infrared-spectrum no formyl peak is present so that it can be said that the product is free of monomer gramicidin. Additionally, it can be concluded on the basis of the absence of an ester peak, that the OH-group on the ethanolamine terminal of the molecule is intact, i.e. the carbon suboxide has reacted selectively with the amino group of the desformylated gramicidin. The absence of an ester peak is also important with respect to the phenolic OH group of the desformylated gramicidin C, which is present in a concentration of approximately 19%.

(3) The findings in point 2 are supported by results obtained by proton resonance spectroscopy. The spectra were taken in a 30% (w/v) solution of malonyl-bis-desformylgramicidin and monomer gramicidin, respectively. The solvent was deuterated dimethylsulfoxide. In general, the NMR-spectrum for malonyl-bis-desformylgramicidin is the same as for the monomer product, except for the small peak of the $-CH_2$-group of the malonyl residue at 6.6 ppm. This finding is in agreement with the data reported by Urry et al. [7]. In detail, the spectrum shows a signal arising from protons of the ethanolamine OH-group. This supports the results of the infrared spectrum. This means that the dimerization occurred only on the amino group. The signal arising from the proton of the OH-group of the phenolic residue of tyrosine of gramicidin C is the same as for the monomer gramicidin C. The signal arising from the indole residue of the tryptophanes is also the same as was found for the monomer molecule. These data are consistent with the ultraviolet-spectrum.

(4) The last test to characterise malonyl-bis-desformylgramicidin was to determine its molecular-weight in an analytical ultracentrifuge. The result obtained for the monomer gramicidin was a value of approximately 1900 g/mol (theoretical value 1850) and for the dimer gramicidin, about 3800 (theoretical value 3830 g/mol). Methanol was used as a solvent. These data are consistent with the molecular weights determined by the LH 20 Sephadex permeation chromatography.

(II) Bilayer experiments

(a) Dilution and biionic potential. Malonyl-bis-desformylgramicidin increases the membrane conductance in a manner similar to that of the monomer gramicidin [7,17]. In order to prove that the molecule forms cation selective channels as the monomer does, and not unspecific pores, the dilution potential and

TABLE I

RESULTS OBTAINED BY POTENTIAL MEASUREMENTS IN PRESENCE OF MALONYL-bis-DES-FORMYLGRAMICIDIN

$T = 25°C$.

Lipid	Dilution potential	
	Electrolyte	$\Delta\Psi$ (mV)
Dioleoyl phosphatidylcholine *n*-decane	1 M/0.1 M NaCl	−58
		−57
		−58
Dioleoyl phosphatidylcholine *n*-decane	1 M/0.1 M CsCl *	−53
		−55
		−54
		−53
	Biionic potential	
Dioleoyl phosphatidylcholine *n*-decane	1.0 M CsCl/1.0 M NaCl	−44
		−42

* For CsCl the activity coefficient was taken into account so that the experimental value for $\Delta\Psi$ in the table represents nearly the theoretical value of the Nernst-potential.

the biionic potential were measured in the presence of univalent electrolytes. In the case of the dilution potential we obtained the theoretical value for a cation induced potential. The gradient across the membrane was 0.1 M versus 1 M solutions. The results are listed in Table I.

The ion specifity with respect to the cations was measured by determination of the biionic potential. One cation was applied to one side of the membrane, and to the other side the other cation. Both types of electrolytes had the same impermeable anion. In such an experimental arrangement the Goldman-equation is applicable in the following form:

$$E = \frac{RT}{F} \cdot \ln \frac{P_1 \cdot C_1}{P_2 \cdot C_2} \tag{2}$$

where P is permeability and C the concentration of the cation. E is the potential difference.

Table I shows the results obtained by measurements of the biionic potentials. Note that the membrane conductance was kept smaller than 10^{-5} S/cm^2 in order to avoid diffusion polarization arising from the unstirred layers. An evaluation of these data discloses a permeability ratio between Cs^+ and Na^+ $P_{Cs^+}/P_{Na^+} = 5.7$. This value is in close agreement with the data which were obtained by Mayers and Haydon [10] for the monomer gramicidin.

(b) Relaxation experiments. Previously it was shown by Bamberg and Läuger [12], that the kinetics of the dimerization of two monomer gramicidin molecules in a lipid membrane can be studied by voltage jump experiments. A voltage jump disturbs the equilibrium between monomers and conducting dimers in the membrane. The equilibrium in the membrane

$$G + G \underset{k_D}{\overset{k_r}{\rightleftharpoons}} G_2 \tag{3}$$

is shifted towards the side of the conducting dimers at higher voltages. According to the dimer model of the channel the membrane current is proportional to the dimer concentration in the membrane. After a voltage jump the current should relax to a higher stationary level. It was successfully demonstrated for several lipid-membrane systems [12—14] that the current $J(t)$ is governed by a single exponential for a wide range of gramicidin A-induced conductances.

$$J(t) = J_\infty + (J_0 - J_\infty) \exp(-t/\tau) \tag{4}$$

where J_0 is the initial and J_∞ the steady-state current. The relaxation time τ is given by

$$\frac{1}{\tau} = k_D + 4\sqrt{\frac{k_r \cdot k_D \lambda^\infty}{L \cdot \Lambda}} \tag{5}$$

where k_D is the dissociation rate constant, k_r the association rate constant for the dimerization process (Eqn. 3), L Avogadro's constant and λ^∞ the steady-state conductance of the membrane, which is reached after a long period of time (t). Λ is the single channel conductance of gramicidin A.

In the case of malonyl-bis-desformylgramicidin, a voltage-induced relaxation of the current should not occur, if malonyl-bis-desformylgramicidin as a chemical dimer produces ionic channels itself.

Figs. 3—5 show voltage jump experiments under similar experimental conditions (lipid, voltage, conductance) in the presence of gramicidin A and malonyl-bis-desformylgramicidin and, as a control experiment, with valinomycin.

Fig. 3a clearly shows a current relaxation after a voltage jump in the presence of malonyl-bis-desformylgramicidin. The analysis of this curve indicates a nonexponential behaviour. After 60 min the experiment was repeated on the same membrane. The result can be seen in Fig. 3b. This time the relaxation amplitude is reduced by a factor of 3.

An explanation of this phenomenon is possibly given by the time dependence of the compressibility of the membrane. It was shown that after 60 min the compressibility of a dioleoyl phosphatidylcholine/*n*-decane membrane is reduced by a factor of more than 3 [31]. Possibly malonyl-bis-desformalgramicidin molecules in the membrane are partially inactive in the absence of an applied voltage, due to geometrical causes. After application of a voltage, the membrane becomes thinner [30,31], so that a portion of the inactive molecules are able to bridge the membrane and can contribute to the conductance. This explanation seems to be reasonable, because, according to Urry, the dimerized molecule has a length of 30 Å, whereas the thickness of the hydrocarbon of a phosphatidylcholine/*n*-decane membrane is formed to be approximately 42 Å [32].

The same relaxation experiments with gramicidin A as described with malonyl-bis-desformylgramicidin are demonstrated in Figs. 4a and b. A reduced amplitude after long intervals was found here, also (Fig. 4b). An evaluation of these experiments (Figs. 4c and d) shows that the relaxation current is governed by a single exponential [12—14]. After long periods of $\geqslant 2$ s a relatively slow increase in current can be observed. A tentative explanation for this behaviour [12] was that the torus became smaller after a voltage jump.

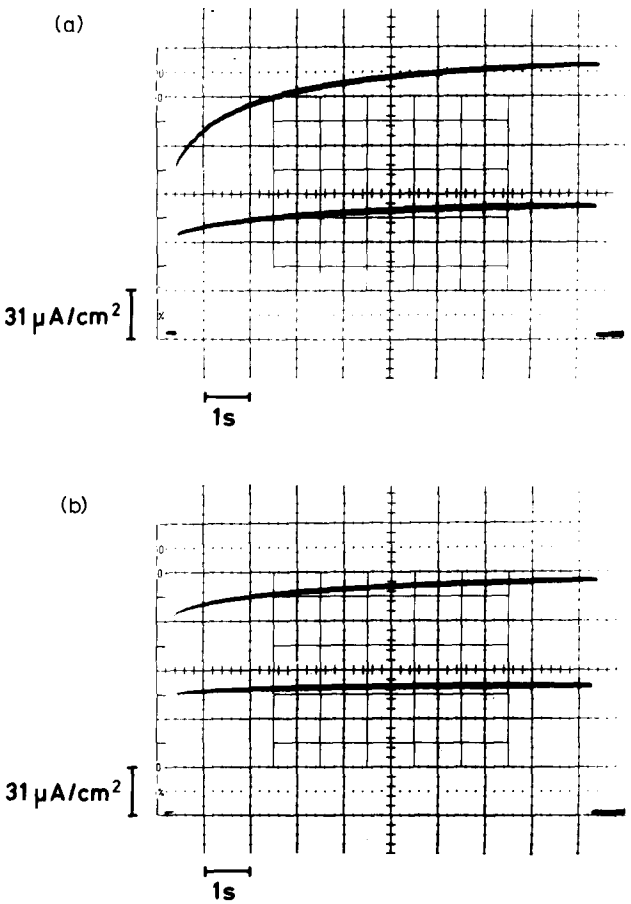

Fig. 3. (a) Current relaxation after a voltage jump in presence of malonyl-bis-desformylgramicidin. Lipid, dioleoyl phosphatidylcholine/*n*-decane (1% w/v); electrolyte, 1 M KCl; $T = 25°C$; $V = 100, 150$ mV. Time after blackening of the membrane 5 min. (b) Identical conditions as described in (a) on the same membrane. Time after blackening of the membrane 60 min.

The torus, however, was kept to a minimum with a membrane area of about $8 \cdot 10^{-2}$ cm^2. It was not greater than 10% of the total membrane area. This means that the slow increase of the current cannot arise solely from a torus effect, because the slow increase, occurring after the blackening of the membrane, is approximately 30% of the total relaxation amplitude. Figs. 4a, and c show clearly a deviation from the single exponential behaviour after the actual relaxation induced by gramicidin A. This deviation is markedly reduced or disappears when the membrane is aged (Figs. 4b and d) and can be discounted.

This means that aside from the active dimerized molecules in the membrane, various inactive dimerized molecules may exist, which are perhaps activated by the thinning of the membrane. Similar arguments are valid for malonyl-bis-desformylgramicidin doped membranes.

Furthermore, the thinning of the membrane can lead to a higher probability that two monomer molecules can react with each other, forming a new channel. Both effects are more probable on a freshly formed membrane. Addition-

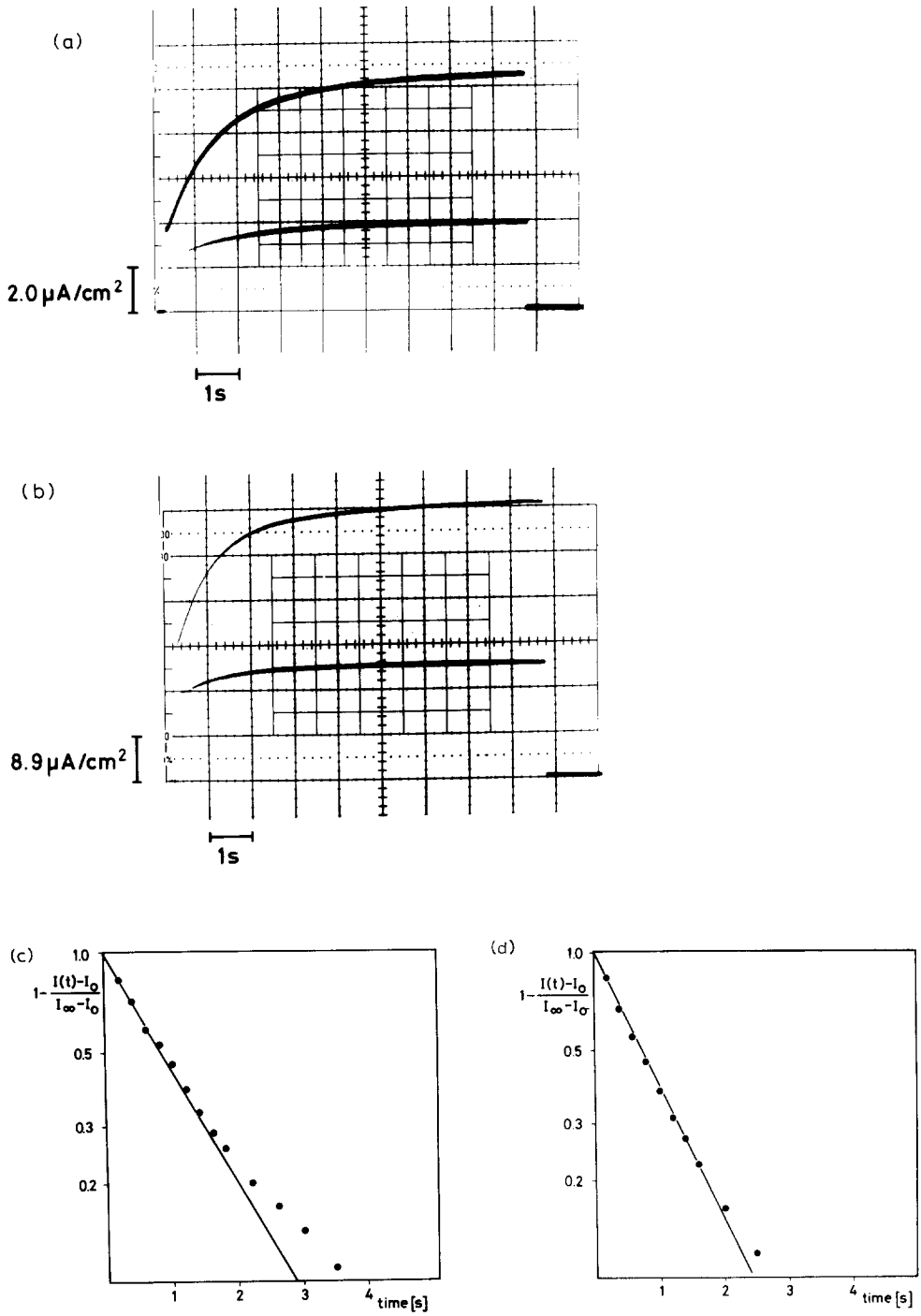

Fig. 4. (a) Current relaxation after a voltage jump in presence of gramicidin A. Lipid, dioleoyl phosphatidylcholine/*n*-decane (1% w/v); electrolyte, 1 M KCl; $T = 25°C$; $V = 100$, 150 mV. Time after blackening of the membrane 5 min. (b) Identical conditions as in (a) on the same membrane. Time after blackening of the membrane 60 min. (c) Evaluation of the current relaxation (a) at 150 mV upper trace. (d) Evaluation of the current relaxation (b) at 150 mV upper trace.

ally, the reduction of the relaxation amplitude over a length of time can be explained partially in the following way.

The model for the dimerization of gramicidin A in a lipid membrane requires that the relaxation amplitude becomes smaller with increasing membrane conductance [12]. At high levels of conductance, i.e. at high concentrations of gramicidin in the membrane, the molecule should be present mainly in the dimerized form, so that the relaxation amplitude becomes smaller with increasing conductance. In the presence of gramicidin A it is impossible, experimentally, to keep the membrane conductance constant over long periods of time ($\geqslant$20—60 min). The experiments represented in Figs. 4a and b show that the conductance is increased within one hour by a factor of approximately 3.5. On the other hand, the increase of the conductance λ^{∞} is much slower than the slow increase of the current after a voltage jump, so that we can exclude this effect as direct relaxation phenomenon.

In order to verify that the above described phenomena were not the result of trivial effects, such as a voltage induced increase of the membrane area at the cost of the torus and the small microlenses [30,31], the current-time behaviour of a valinomycin-doped membrane ($2 \cdot 10^{-3}$ M valinomycin in the lipid phase) was measured.

Fig. 5 clearly shows that such an alteration in the area is very small, and occurs faster by a factor of about 3, compared to the gramicidin A relaxation. It should be noted that the rapid valinomycin relaxation is not resolved in this figure [33]. In contrast to the gramicidin the thickness change of the membrane does not alter the transport rate of the valinomycin K^+ system markedly.

(c) Single channel experiments. The properties of malonyl-bis-desformyl-gramicidin channels were measured for different lipid systems. Fig. 6a shows a typical experiment, whereas in Fig. 6b the result is compared with the current fluctuation arising from appearing and disappearing gramicidin A channels. The conditions for both experiments were identical (lipid, electrolyte and temperature). In most of the experiments 10% cholesterol was added to the lipid

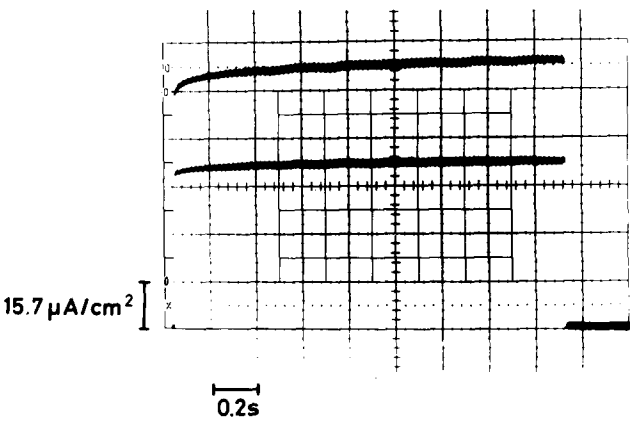

Fig. 5. Current relaxation after a voltage jump in the presence of $2 \cdot 10^{-3}$ M valinomycin in the lipid-phase. Lipid, dioleoyl phosphatidylcholine/*n*-decane (1% w/v); electrolyte, 1 M KCl; $T = 25^{\circ}$C; $V = 100$ mV; $V = 150$ mV. Time after blackening of the membrane 20 min.

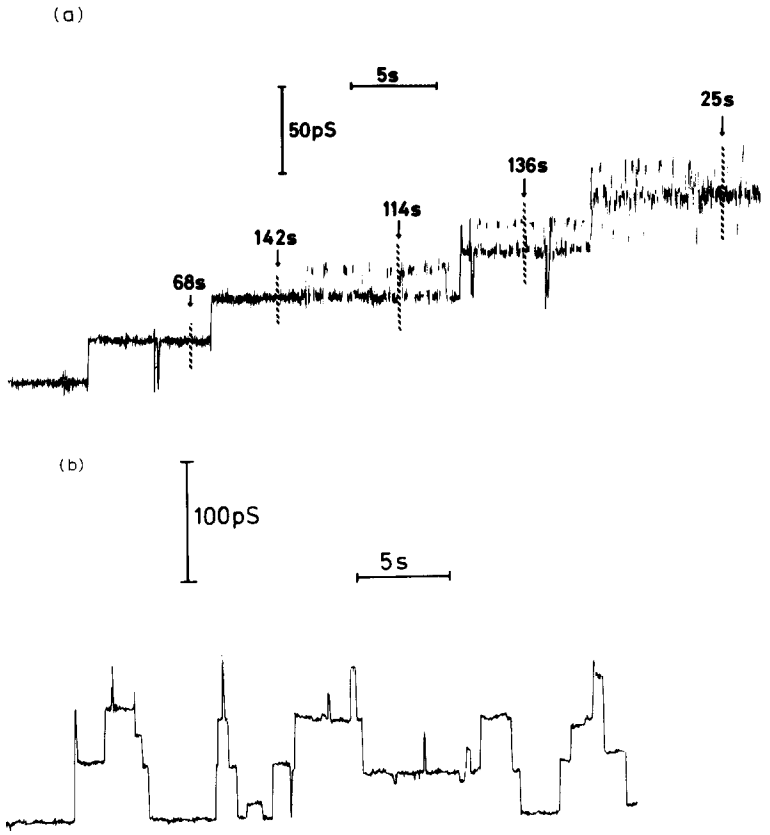

Fig. 6. (a) Single channel response of malonyl-bis-desformylgramicidin. Lipid, diphytanoyl phosphatidylcholine/cholesterol 0.9% + 0.1%/*n*-decane; electrolyte, 1 M CsCl; $T = 25°C$; $V = 100$ mV. (b) Single channel response of gramicidin A. Identical conditions as described in (a).

w/w. Membranes made from such a mixture have a lower basic conductance level, making it possible to avoid unspecific current fluctuations, which can arise after longer period of time (10—20 min), as can be seen especially in the case of dioleoyl phosphatidylcholine and monoolein membranes. In the presence of malonyl-bis-desformylgramicidin it was necessary to carry out measurements over very long time periods because of the long single channel life times.

Furthermore, malonyl-bis-desformylgramicidin seems to introduce a "noise" to the membrane, which can impair the measurements. This "noise" can be reduced by the addition of cholesterol to the membrane. It is known however, from the potential measurements described in IIa of this paper, that the ion selectivity seems not to be influenced by this effect.

The single channel conductance of malonyl-bis-desformylgramicidin is markedly reduced compared with the monomer gramicidin A. It is impossible to give an exact value arising from the usual statistical evaluation of the single channel events, because the life time of the single channels is very long. The variation of single channel conductance on the same membrane system was

TABLE II

SINGLE CHANNEL CONDUCTANCE AND LIFE TIME IN PRESENCE OF MALONYL-bis-DESFORMYLGRAMICIDIN AND OF GRAMICIDIN A

In the case of gramicidin A Λ and the mean duration of the single channels τ^* were obtained by statistical evaluation [9,13]. In the case of malonyl-bis-desformylgramicidin approximately 20 channels for each system were evaluated due to the long duration of the channels. MBDG, malonyl-bis-desformylgramicidin. The electrolyte on all experiments was 1 M CsCl; $T = 25^\circ C$.

Lipid	Λ g A(pS)	Λ_{MBDG}(pS)	τ * gA(S)	Life time of differen MBDG-channels (s)
Diphytanoyl phosphatidylcholine/cholesterol/*n*-decane (0.9/0.1%, w/v)	29	14—19	1.7	≈200
Dioleoyl phosphatidylethanolamine/cholesterol/*n*-decane (0.9/0.1%, w/v)	45	14—23	1.5	≈350
Monoolein/cholesterol/*n*-decane (1.8/0.2%/*n*-decane, w/v)	—	20—36	—	≈ 80

within a factor 2—3. The same phenomenon is found for monomer gramicidin (Fig. 6b). The explanation for varying amplitudes could be found in the heterogeneity of the gramicidins we used for the dimerization, but it seems quite improbable, due to the fact that different amplitudes were found even with purified gramicidin A [34]. A tentative explanation for the appearence of different amplitudes was given previously. There may not be a single channel but a range of different hydrogen bounded structures, and one of these structures may be "frozen" in the lipid matrix [34]. This seems reasonable, because within the extremely long life time of the malonyl-bis-desformylgramicidin channels a change of the current amplitude of the same pore could not be detected. In Table II the single channel conductance of malonyl-bis-desformylgramicidin is listed for membranes made from different lipids. The electrolyte was 1 M CsCl. For each systems about 15—25 channel events were evaluated.

To understand the mechanism, it is more interesting to study the kinetic behaviour of malonyl-bis-desformylgramicidin channels on the single channel basis than the single channel conductance. The single channel life time is dramatically increased compared with the monomer gramicidin A. This result is expected on the basis of the dimer hypothesis. According to Urry's model [7], a chemically dimerized molecule should produce channels with a very long life time, since the malonyl-bis-desformylgramicidin cannot switch off, due to dissociation. The inactivation of such a channel can only occur by its diffusion into a part of the membrane, where, for geometrical reasons (torus, small lenses, more solvent containing regions of the membrane [30,31]), the channel cannot exist in the conducting form.

Another remarkable phenomenon is shown in Fig. 6a, the rapid switching on and off of probably the same channel. This fluctuation could arise from the same channel and presumably is not due to the association and dissociation of two dimer molecules to form a tetramer, producing new channels. This could be possible, according to a proposal by Veatch and Blout [18]. These authors

propose an alternate channel model to the hypothesis by Urry. They postulate that two monomer gramicidin molecules can form a parallel and an antiparallel helix, respectively. This is valid, too, for the chemically linked molecule. Other recent sources, as well as this paper have reported that different channels have slightly different current amplitudes [9,13,34]. In fact, if the rapid switching on and off arises from the appearance and disappearance of different channels within the switching different amplitudes and a superposition of such channels should be seen. Both phenomena are not detectable (Fig. 6a). A tentative explanation for this behaviour could be found in different structural effects on the membrane. Possibly the channel is in a border region, where the molecule can bridge the membrane. Just a very small change of the local membrane thickness can switch off the channel, so that a minimal fluctuation of the membrane thickness could be responsible for the conducting or the nonconducting state of the channel. A superposition of two or three malonyl-bis-desformylgramicidin channels results in current fluctuations which cannot be resolved by single channel experiments (Fig. 6a). Furthermore, from the absence of a characteristic relaxation it seems rather unlikely that two malonyl-bis-desformylgramicidin molecules could form a new channel.

A second explanation is possibly given by an arrangement of the molecule according to the proposal by Veatch and Blout [18]. This signifies that the molecule is folded on the chemical linkage, so that a parallel structure should be possible. Then a fast association and dissociation of the two parts of the molecule could occur and could be responsible for the fast switching. But this interpretation is rather unlikely because a negatively charged monomer gramicidin is inactive [35] if it is applied only on one side of the membrane, whereas the activity of unmodified gramicidin A is not different if applied to one or both sides of the membrane.

In membranes containing cholesterol, which are presumably more rigid with respect to the structure, malonyl-bis-desformylgramicidin does not exhibit such a rapid switching effect. In addition to the single channel amplitudes Table II contains the evaluation of some single channel life times. Here the rapid switching was not taken into account, assuming that the same channel shows this effect. Here an exact statistical evaluation is not possible due to the very long channel life time. These results are not in agreement with the data obtained by Goodall [11]. He found a mean life time in the range of 1—2 s for succinyl-bis-desformylgramicidin, whereas in the experiments described here the channel lifetime was in the range of at least 100—200 s. The discrepancy could possibly have been caused by an impurity of the product. This signifies that a mixture of monomer and dimer gramicidins can lead to the findings which are presented in Goodall's paper.

On the basis of the experiments described above it seems reasonable to assume that malonyl-bis-desformylgramicidin can form ionic channels itself without further association with another molecule in the membrane.

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