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Structure of the gramicidin A channel: Discrimination between the $\pi_{L,D}$ and the β helix by electrical measurements with lipid bilayer membranes

(ionic channels/gramicidin A analogs/channel structure)

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ABSTRACT **Measurements with different chemically modified gramicidins in lipid bilayer membranes were used to discriminate between the dimeric $\pi_{L,D}$ helix proposed by Urry and the dimeric parallel or antiparallel helices proposed by Veatch and Blout. Evidence for the $\pi_{L,D}$ helix was obtained on the basis of the different actions of a negatively charged *O*-pyromellitylgramicidin and a negatively charged *N*-pyromellityldesformylgramicidin on lipid bilayer membranes. *O*-Pyromellitylgramicidin forms ionic channels in lipid membranes when it is applied to both sides of the membrane. In contrast to unmodified gramicidin, *O*-pyromellitylgramicidin is inactive when it is applied only to one side of the membrane. *N*-Pyromellityldesformylgramicidin does not form ionic channels in lipid bilayer membranes whether it is applied to one or both sides of the membrane. These results support the view that the gramicidin channel is formed by two $\pi_{L,D}$ helices. Dimer formation by head-to-head association of two $\pi_{L,D}$ helices needs six intermolecular hydrogen bonds, which are located at the formyl end of the molecule and which occur deep within the lipid membrane. In the head-to-head associated $\pi_{L,D}$ helix the absence of the formyl group leads to an inactivation of the peptide, whereas in a parallel or antiparallel double-stranded helix the absence of the formyl group should have only minor effects.**

Gramicidin A is a linear pentadecapeptide consisting of 15 hydrophobic alternating D,L-amino acids. It induces, in natural membranes and artificial lipid membranes, a high permeability for small monovalent cations. There is strong evidence that the peptide forms pore-like channels (1–12). For the structure of the channel two proposals exist. One possibility is a head-to-head association (formyl end to formyl end) of two helical monomers, where the helix of the monomer is a $\pi_{L,D}$-helix (7, 13).

The helix in the dimeric form is stabilized by 22 intramolecular and 6 intermolecular hydrogen bonds. Urry *et al.* (7) proposed four types of $\pi_{L,D}$ helices, where the $\pi^6_{L,D}$helix seems to be the most probable. With the length of that dimer (30 Å) and the diameter of the hydrophilic hole (4 Å), the best geometrical dimensions are obtained to explain the results gotten in the membrane experiments. The other possible $\pi_{L,D}$ helices either are too short ($\pi^8_{L,D}$ and $\pi^{10}_{L,D}$ helices) to form a transmembrane channel or the pore size is too small (1.8 Å diameter for $\pi^4_{D,L}$ helix).

An alternate model, a double-stranded helix, in which both peptide chains are coiled around a common axis, has been proposed by Veatch *et al.* (14, 15). In contrast to the $\pi_{L,D}$ helices, these helices have no intramolecular hydrogen bonds but 20–28 intermolecular hydrogen bonds (14). A common feature of both models is the existence of a narrow pore along the axis of the dimer that is lined with oxygen atoms of the peptide carbonyls. The essential difference between both models is that the $\pi_{L,D}$ helices dimerize when the formyl group is intact so that the intermolecular hydrogen bonds can form a bridge between two molecules. For the double helix this premise is not necessary. Some experimental hints that the $\pi_{L,D}$ helix is the transmembrane channel are given by Goodall (6) and Urry *et al.* (16). These authors found that *N*-acetyldesformylgramicidin and *N*-*t*-butyloxycarbonylgramicidin show a markedly lower activity on membranes than the unmodified gramicidin A. The dimerization constant, however, for *N*-*t*-butyloxycarbonylgramicidin is, in solution, the same as for the unmodified gramicidin. From these findings Urry *et al.* (16) concluded that "the dominant aggregation seen at higher concentrations and elevated temperatures is not directly relevant to the channel formation in the black lipid membrane studies."

This paper describes the action of a negatively charged gramicidin A analog, *N*-pyromellityldesformylgramicidin (Fig. 1*a*). The results are compared with those obtained previously (17) with another type of negatively charged gramicidin, *O*-pyromellitylgramicidin (Fig. 1*b*). Due to the free negative charges of the pyromellityl residue at the amino group or at the ethanolamine group, the molecule is unable to pass through the membrane from one side to the other side as unmodified gramicidin presumably does. That means the negative charges force the desformylated end of the *N*-pyromellityldesformylgramicidin and the ethanolamine end of the *O*-pyromellitylgramicidin to remain always at that membrane interface where the substance was applied. Therefore, it should be possible, on the basis of different activities of the two different modified gramicidins, to decide between both proposals for the channel structure.

The observed activity of *O*-pyromellitylgramicidin can be explained on the basis of a double-stranded helix as well on the basis of a $\pi_{L,D}$ helix (17). *N*-Pyromellityldesformylgramicidin can be active as a double-stranded helix. A $\pi_{L,D}$ helix dimer, formed by head-to-head association, must be inactive because the *N*-formyl end is blocked and located in the membrane interface. Besides the experimental advantages in membrane experiments, the negatively charged gramicidins have a further important advantage: the separation of these analogs is much easier than the purification of different neutral gramicidin analogs (17, 18).

MATERIALS AND METHODS

N,*O*-Bispyromellityldesformylgramicidin was synthesized by reaction of desformylgramicidin with pyromelliticdianhydride. Partial alkaline hydrolysis produces *N*-pyromellityldesformylgramicidin. Desformylgramicidin was prepared by a modified method based on the procedure reported by Ishii and Wittkop (19). The starting product was the commercially available "gramicidin pure" from Nutritional Biochemicals Corporation (Cleveland, Ohio). *O*-Pyromellitylgramicidin was prepared as described before (17). Optically black lipid membranes were formed in the usual way from a 1 to 2%

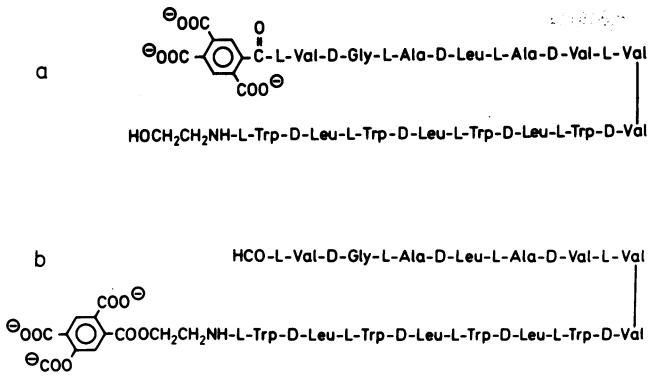

FIG. 1. Structure of *N*-pyromellityldesformylgramicidin (*a*) and of *O*-pyromellitylgramicidin (*b*).

(wt/vol) lipid solution in *n*-decane in a thermostated Teflon cell filled with electrolyte solution (20). The pH was maintained in all experiments at approximately 6.0. The temperature was 25° if not mentioned especially. The membrane area was 3×10^{-2} cm^2 for measurements of the macroscopic conductance and approximately 10^{-3} cm^2 for single channel experiments. Dioleoyllecithin was synthesized in our laboratory by K. Janko. Monoolein was obtained from NuChek Preparation Corp., Elysian, MN, and was used without further purification. For single channel experiments, the same setup was used as described earlier (21).

RESULTS

Preparation of *N*-pyromellityldesformylgramicidin

For the desformylation reaction a solution of 0.5 g of gramicidin A in 50 ml of 1.5 M absolute methanolic HCl was kept at 30° for 3 hr under nitrogen. Subsequently the HCl was neutralized by an equivalent amount of NaOH. The mixture was diluted with water to a volume of 200 ml and the precipitated desformylgramicidin was separated by centrifugation. The methanolic solution of the precipitate was evaporated and the remaining solid dried over phosphorus pentoxide, yielding 0.45–0.47 g of solid product. The melting point of the product was 200–205°. Thin-layer chromatography and infrared spectroscopy showed no difference from desformylgramicidin made and purified by the method of Ishii and Wittkop (19). The homogeneity and the molecular weight were checked by sedimentation analysis on an ultracentrifuge (Beckman ultracentrifuge type E). The estimated molecular weight was 1850 (theoretical value, 1820). To exclude traces of unreacted gramicidin, we purified desformylgramicidin by thin-layer chromatography on silica gel S plates using the solvent chloroform/methanol/water (65:25:4, vol/vol).

N,O-Bispyromellityldesformylgramicidin was prepared in the following way. A solution of 0.3 g of desformylgramicidin and 2 g of pyromellitic acid dianhydride was kept at 40° in 10 ml of absolute pyridine under nitrogen for 24 hr. Then the pyridine was evaporated under reduced pressure and the solid residue dissolved in a solution of 3.0 g of sodium bicarbonate in 50 ml of water. After dilution with 500 ml of 2% aqueous NaCl and cooling to 0–5° for several hours, the precipitated sodium salt of *N,O*-bispyromellityldesformylgramicidin was separated by centrifugation. The sediment was redissolved in 10 ml of methanol; after filtration from the undissolved material, the precipitation was repeated. The resulting product (about 0.5–0.20 g) was dried under reduced pressure over phosphorus pentoxide. In a test with thin-layer chromatography using chloroform/methanol/water (65:25:4, vol/vol) on silica gel S plates, the product remained at the starting point, The infrared spectrum showed the known gramicidin spectrum with an additional ester carbonyl peak at 5.8 μm.

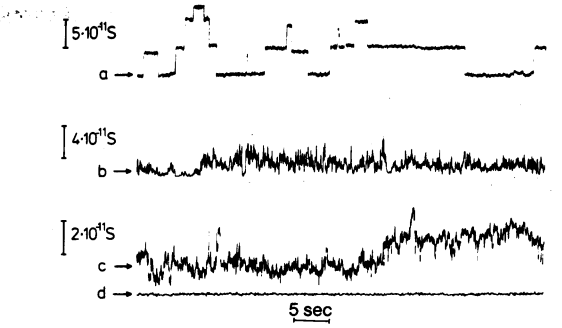

FIG. 2. Single channel behavior in the presence of *O*-pyromellitylgramicidin (*a*) and *N*-pyromellityldesformylgramicidin (*b* and *c*). Trace *a*: monoolein/*n*-decane (2% wt/vol) as lipid solution. 0.2 M CsCl; $t = 25°$; $V = 100$ mV. Trace *b*: 1 M $CaCl_2$. Other conditions as in trace *a*. Trace *c*: 0.2 M CsCl. Other conditions as in trace *a*. Trace *d*: current record from the same membrane before *N*-pyromellityldesformylgramicidin (trace *c*) was added.

To obtain *N*-pyromellityldesformylgramicidin we added the *N,O*-bispyromellityldesformylgramicidin to a solution of 0.1 M NaOH in 10 ml of methanol. After this solution was kept for 10 hr at 20° under nitrogen, the mixture was acidified with 1 M HCl to pH 1 and diluted with water to 100 ml. The precipitated product was separated by centrifugation. After evaporation of the solvent and drying over phosphorus pentoxide, 84 mg of *N*-pyromellityldesformylgramicidin were obtained in the free acid form.

With the final product the following analytical tests were carried out. The UV spectrum showed the typical absorbance of the tryptophan at 280 nm with a molar extinction coefficient of $\epsilon = 22{,}400$, calculated for a molecular weight of 2057. A parallel run with unmodified gramicidin A gave a value for ϵ of approximately 21,500. The sodium salt of the *N*-pyromellityldesformylgramicidin was obtained by neutralizing the methanolic solution of the free acid with sodium bicarbonate.

Thin-layer chromatography was performed on coated silica gel S plates with two different solvent mixtures: chloroform/methanol/water (65:25:4, vol/vol) and *n*-butanol/acetic acid/water (63:27:10, vol/vol). Traces of pyromellitic acid and nonpolar impurities were removed by preparative thin layer chromatography. The infrared spectrum showed the absence of the estercarbonyl absorption at 5.8 nm.

The molecular weight was estimated by sedimentation analysis with an analytical ultracentrifuge. Under the conditions reported before (17), the estimated molecular weight was 1900 (theoretical value, 2057).

Membrane experiments

Single Channel Experiments. In Fig. 2 are shown the single channel responses induced by *O*-pyromellitylgramicidin in the presence of 0.2 M CsCl (trace *a*) and by *N*-pyromellityldesformylgramicidin in the presence of 1 M $CaCl_2$ (trace *b*) and 0.2 M CsCl (trace *c*). As a control the trace of the unmodified membrane is shown in trace *d*. The experiments indicate clearly that *N*-pyromellityldesformylgramicidin acts in a different manner from *O*-pyromellitylgramicidin or unmodified gramicidin A. *N*-Pyromellityldesformylgramicidin does not produce the typical current fluctuations that may be seen, for example, in the presence of *O*-pyromellitylgramicidin (Fig. 2, trace *a*). *N*-Pyromellityldesformylgramicidin acts in the

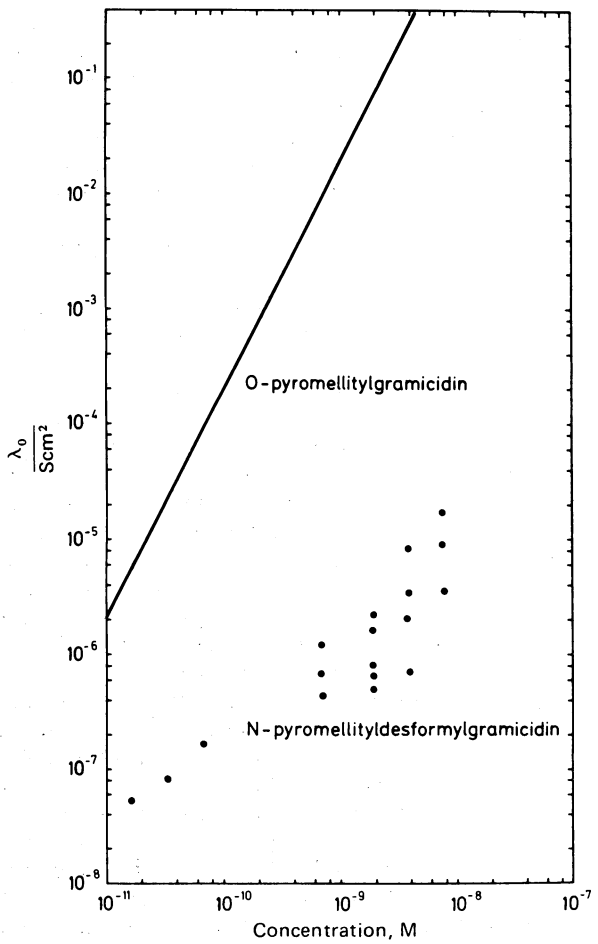

FIG. 3. Steady-state conductance in the presence of *N*-pyromellitylgramicidin. Lipid: dioleoyllecithin/*n*-decane (2% wt/vol); 0.2 M CsCl; $t = 22°$, $V = 25$ mV. For comparison, the data obtained with *O*-pyromellitylgramicidin (under identical conditions) were taken from a previous publication (ref. 17).

membrane if added in concentrations higher than 10^{-10} M to one or both sides of the membrane. This result is in contrast to the result with *O*-pyromellitylgramicidin, which is only active when applied to both sides of the membrane. A further important difference in the behavior is that the membrane conductance is also increased in the presence of $CaCl_2$, a result that was never found with unmodified gramicidin A or *O*-pyromellitylgramicidin (9, 17). The critical concentration of *N*-pyromellitylgramicidin has to be 10^3 times higher than that of *O*-pyromellitylgramicidin to produce any effect on the membrane. When *N*-pyromellitylgramicidin was added to the solution in a concentration that was 10^5–10^6 times higher than the concentration used in the single channel experiments with unmodified gramicidin A, some gramicidin A channels were observed. This indicates that the molar fraction of unmodified gramicidin A in the sample of *N*-pyromellitylgramicidin that we have used is of the order of 10^{-6}–10^{-5}.

All these results together lead to the conclusion that *N*-pyromellitylgramicidin acts as a detergent rather than as a channel former. This is also indicated by the shape of the current fluctuations.

In a second series of single channel experiments, we tried to form hybrid channels in the following way: to one side of the membrane an amount of *O*-pyromellitylgramicidin was added. Then an approximately 10^3 times higher concentration of *N*-

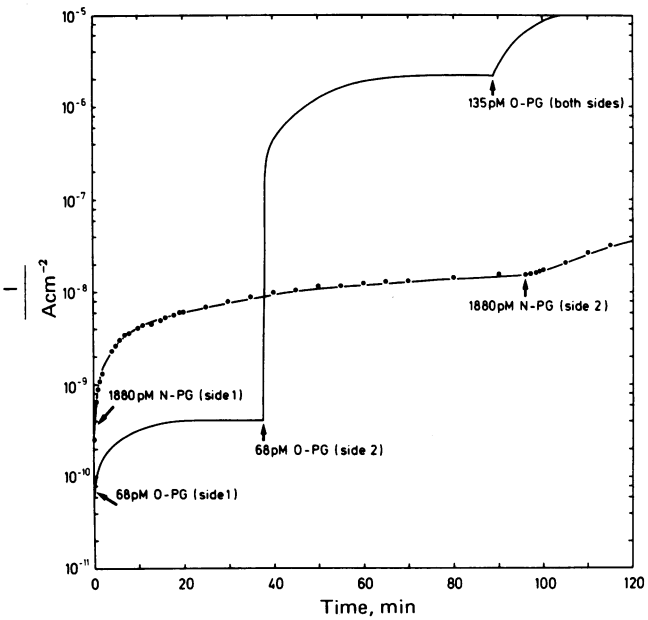

FIG. 4. Membrane current after asymmetric and symmetric addition of *N*-pyromellitylgramicidin (N-PG). Both aqueous solutions contained 0.2 M CsCl. $t = 22°$, $V = 25$ mV. For comparison, the data obtained with *O*-pyromellitylgramicidin (O-PG) under identical conditions were taken from a previous publication (ref. 17).

pyromellitylgramicidin was added to the other side of the membrane. The result was a noisy current trace of the type represented in Fig. 2, trace *c*. After destruction of the membrane a new membrane was formed after some mixing of the bath solutions; immediately, typical *O*-pyromellitylgramicidin channels appeared. These findings demonstrate clearly that hybrid channels between *N*- and *O*-pyromellitylgramicidin cannot occur.

Multichannel Experiments. Previously it was shown that in the presence of *O*-pyromellitylgramicidin it was possible to measure the steady-state conductance of a membrane as a function of the concentration of the peptide (17). Fig. 3 shows a similar experiment with *N*-pyromellitylgramicidin; for comparison, the previous experiments obtained with *O*-pyromellitylgramicidin are also included in the figure. With *O*-pyromellitylgramicidin the membrane conductance increases with the square of the peptide concentration (17). The result of the same experiment with *N*-pyromellitylgramicidin is quite different. In a double-logarithmic plot the slope is smaller than 1 and the absolute conductance values are much smaller than those observed with *O*-pyromellitylgramicidin. In addition, the reproducibility of these experiments is poor (Fig. 3). At concentrations greater than 10^{-8} M the membrane stability became very low.

These facts agree with the above-mentioned detergent effect of the molecule. A second series of multichannel experiments was done to investigate the time behavior of the membrane conductance after addition of *N*-pyromellitylgramicidin. To get a distinct increase of the membrane conductance, we added *N*-pyromellitylgramicidin to a concentration of 1800 pM to one side of the membrane. In approximately 60 min the current increased by one order of magnitude. The addition of the same amount to the other side of the membrane resulted in a further small increase of the conductance (Fig. 4).

The completely different behavior of *O*-pyromellitylgramicidin, as described previously (17), is also shown in Fig.

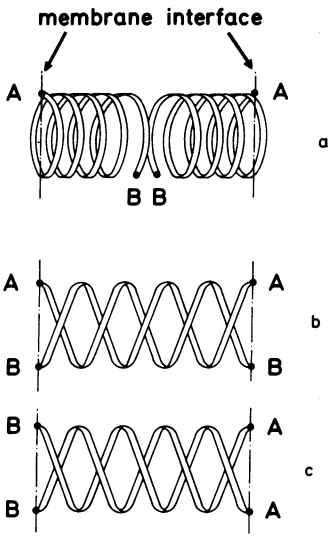

FIG. 5. Schematic representation of the molecular arrangement of the gramicidin channel in the membrane. (*a*) The $\pi_{L,D}$ helix according to Urry (7, 13). The antiparallel (*b*) and the parallel (*c*) formation of a double-stranded helix according to Veatch *et al.* (14, 15).

A stands for the modified or unmodified ethanolamine residue.

A: —NH—CH_2—CH_2—OH

or —NH—CH_2—CH_2—OOC— (benzene ring) —COO^-, ^-OOC— —COO^-

B stands for the formyl end of the molecule or for the *N*-pyromellitic group of the desformylated end of the molecule.

B: —NH—CHO

or —NH—OC— (benzene ring) —COO^-, ^-OOC— —COO^-

4. The addition of *O*-pyromellitylgramicidin to one side produces only a small increase of the conductance, whereas the addition of the same amount (68 pM) of the peptide to the other side of the membrane induces a conductance change of several orders of magnitude. A more detailed description of the action of *O*-pyromellitylgramicidin is presented in a previous publication (17).

DISCUSSION

The single channel and multichannel experiments show clearly that *N*-pyromellityldesformylgramicidin is not able to form ionic channels in lipid bilayer membranes. This result, combined with previous experiments carried out with a chemically linked malonylbisdesformylgramicidin (18) and with *O*-pyromellitylgramicidin (17), allows us to decide between the different proposals for the channel structure in the membrane.

The Double-Stranded Helix. Veatch *et al.* (14, 15) proposed a double-stranded helix on the basis of spectroscopic studies of gramicidin in solvents of different polarity. They distinguished between two different conformations of a dimeric helix: a parallel (Fig. 5*b*) and an antiparallel (Fig. 5*c*) form.

Within the framework of the model of Blout and Veatch it is difficult to assign to the *N*-formyl group any critical role in channel formation. In other words, in this model desformylated gramicidins should form channels too, and the pyromellitic residue should not impede pore formation. The fact that the charged group compels the *O*-pyromellitylgramicidin and the *N*-pyromellityldesformylgramicidin to be in defined alignments in the membrane leads to the following conclusions. The application of *N*-pyromellityldesformyl- or *O*-pyromellitylgramicidin to only one side of the membrane would enable the parallel double helix to be formed (Fig. 5*c*) if the electrostatic repulsion of the charged groups is not too strong. This repulsion cannot occur when *O*-pyromellitylgramicidin is applied to one side of the membrane and *N*-pyromellityldesformylgramicidin to the other. None of these experiments shows channel-like fluctuations. This result excludes the parallel helix as a possible structure of the active channel. Whereas the model of Urry excludes channels that cross membranes when the formyl group is missing, in the double-stranded helical model it is possible to form an antiparallel helix, built up by two *N*-pyromellityldesformylgramicidins, and only this configuration is feasible. The experimental result, however, is negative; no channels could be found.

The $\pi_{L,D}$ Helix. Urry's model is based on the formation of a dimer in the $\pi_{L,D}$ helical arrangement shown in Fig. 5*a*. This model requires a head-to-head association by six hydrogen bonds at the *N*-formyl end of the molecules. Therefore, gramicidin derivatives have to be inactive in this model if the *N*-formyl group is replaced by another group that prevents the hydrogen bond formation. Especially when a charged group forces this end of the molecule to the membrane–solution interface, no channels can occur. When the pyromellityl residue is linked to the ethanolamine end of the gramicidin, this molecule should be active according to this model.

The experimental results are in agreement with this model. In experiments with *N*-pyromellityldesformylgramicidin, no channels were formed; even the hybrid experiments failed (*N*-pyromellityldesformylgramicidin on one side of the membrane and *O*-pyromellitylgramicidin on the other side). This result demonstrates that the presence of two *N*-formyl groups is crucial for dimerization. Only if the *N*-formyl groups of both molecules are able to combine can a channel be formed, whether or not there is a modified ethanolamine end of the molecule (17).

The value of the activation energy of about 17 kcal/mol (9, 21) for the dissociation of a dimer in the membrane is also consistent with the Urry model. When six hydrogen bonds are responsible for the dimerization process in the membrane, then a value of 3 kcal/mol per one hydrogen bond is reasonable for hydrogen bonds in an apolar medium like that of the interior of a lipid membrane. An additional interpretation of the high value of the activation energy of the association rate constant (20 kcal/mol) could be the existence of a preequilibrium between the inactive double-stranded β helix and the active $\pi_{L,D}$ helix in the membrane. This possibility is supported by the existence of the double-stranded β helix in different solvents (14).

A further argument for the $\pi_{L,D}$ helix is given by the kinetic behavior of chemically linked gramicidin dimer. Malonylbisdesformylgramicidin forms ionic channels in lipid membranes (7, 18), but the mean lifetime of the channel is in the range of minutes, whereas the channel formed by two monomers has a lifetime of the order of seconds in the same lipid (18). Furthermore, the chemically linked dimer does not produce the typical electrical relaxation phenomena, as described previously (11, 17, 21), which reflect the dimerization process of gramicidin in the membrane.

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