Formation of ion channels by a negatively charged analog of Gramicidin A

ARTICLE *in* JOURNAL OF MEMBRANE BIOLOGY · MARCH 1977

Impact Factor: 2.46 · DOI: 10.1007/BF01869403 · Source: PubMed

4 AUTHORS, INCLUDING:

Hans-Jürgen Apell
Universität Konstanz
161 PUBLICATIONS **3,461** CITATIONS

SEE PROFILE

J. Membrane Biol. 31, 171–188 (1977)

Formation of Ion Channels by a Negatively Charged Analog of Gramicidin A

H.-J. Apell, E. Bamberg, H. Alpes, and P. Läuger

Department of Biology, University of Konstanz, D-7750 Konstanz, Germany

Received 19 July 1976

Summary. O-pyromellitylgramicidin is a derivative of gramicidin in which three carboxyl groups are introduced at the terminal hydroxyl end of the peptide. Experiments with artificial lipid membranes indicate that this negatively charged analog forms ion-permeable channels in a way similar to that of gramicidin. If O-pyromellitylgramicidin is added to only one aqueous solution, the membrane conductance remains small, but increases by several orders of magnitude if the same amount is also added to the other side. In accordance with the dimer model of the channel, the membrane conductance under symmetrical conditions is proportional to the square of the aqueous concentration of O-pyromellitylgramicidin over a wide range. The ratio Λ_{PG}/Λ_G of the single-channel conductance of O-pyromellitylgramicidin to that of gramicidin is close to unity at high ionic strength, but increases more than fivefold at smaller ionic strength (0.01 M). This observation is explained in terms of an electrostatic effect of the fixed negative charges localized near the mouth of the channel. In a mixture of O-pyromellitylgramicidin and gramicidin, unit conductance steps of intermediate size are observed in addition to the conductance steps corresponding to the pure compounds, indicating the formation of hybrid channels. Hybrid channels with preferred orientation may be formed if small amounts of gramicidin and O-pyromellitylgramicidin are added to opposite sides of the membrane. These hybrid channels show a distinct asymmetry in the current-voltage characteristic.

The linear pentadecapeptide gramicidin A has been used in recent years as a model compound for the study of cation transport through hydrophilic channels (Hladky & Haydon, 1970; for a recent survey of the literature, *see* Bamberg, Kolb & Läuger, 1976). There is evidence from electrical relaxation studies (Bamberg & Läuger, 1973, 1974; Bamberg & Benz, 1976), noise analysis (Zingsheim & Neher, 1974; Kolb, Läuger & Bamberg, 1975) and fluorescence measurements (Veatch, Mathies, Eisenberg & Stryer, 1976) that the channel is a dimer of gramicidin A. Two different models for the structure of the dimeric channel have been proposed. The model of Urry (1971, 1972) consists of a helical dimer which is formed by head-to-head (formyl end to formyl end) association of two gramicidin monomers, and which is stabilized by

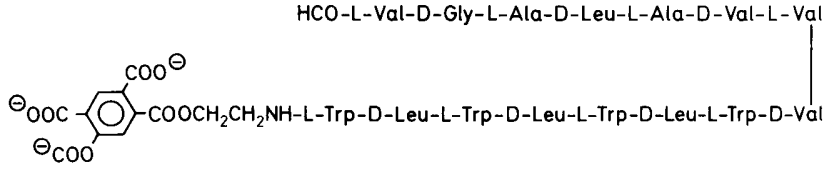

Fig. 1. Structure of O-pyromellitylgramicidin A

intra- and intermolecular hydrogen bonds. In the alternative model of Veatch, Fossel and Blout (1974) the two peptide chains are coiled about a common axis and all hydrogen bonds are intermolecular. At the moment, the experimental evidence is not sufficient to exclude one of these structures. Common to both models is the presence of a 0.3–0.4 nm wide hydrophilic pore along the axis of the dimer which is lined by the carbonyl oxygens of the amide groups.

One of the main goals in studying model channels is a better understanding of the relationship between the chemical structure of the channel and its transport properties. A possible approach to this problem consists in investigating chemical analogs of channel-forming compounds (Ovchinnikov, 1972; Bamberg, Noda, Gross & Läuger, 1975). In this paper we report on the preparation and some of the properties of O-pyromellitylgramicidin A (Fig. 1), a negatively charged derivative of gramicidin A. The study of ion channels with electric charges near the channel mouth seems particularly interesting in view of the observed effects of pH on the conductance of sodium channels in nerve which indicate that the access of ions to the channel is controlled by at least one negatively charged group (Woodhull, 1973; Drouin & Neumcke, 1974; Hille, Woodhull & Shapiro, 1975).

Materials and Methods

O-pyromellitylgramicidin was synthetized by reaction of gramicidin with pyromellitic acid anhydride. As a starting product we used either the commercial gramicidin mixture from Nutritional Biochemicals Corp. (Cleveland, Ohio) which contains approximately 72% gramicidin A, 9% gramicidin B, and 19% gramicidin C, or purified gramicidin A which was a gift of Dr. E. Gross, NIH, Bethesda. 0.4 g commercial gramicidin and 2 g pyromellitic acid anhydride (Fluka AG, Buchs, Switzerland) were dissolved in 10 ml of absolute pyridine and were kept for 48 hr at 20–25 °C under nitrogen. 3.0 g of $NaHCO_3$ in 50 ml water were added dropwise into the red colored solution with stirring. The resulting mixture had a pH of 7–7.5 and contained some undissolved material. After dilution with 500 ml 2% aqueous NaCl and cooling to 0–5 °C for several hr, the sodium salt of O-pyromellitylgramicidin precipitated and was separated by centrifugation. The sediment was redissolved in 10 ml methanol; after separation from a small amount of undissolved material, the precipitation was repeated. The raw product (about 0.3 g) which still contained traces

of pyromellitic acid was further purified by preparative thin-layer chromatography on silicagel 60 plates (Merck, Darmstadt) with chloroform/methanol/water 65:25:4 (v/v). In this solvent system O-pyromellitylgramicidin had a R_F value of 15–20 and was easily separated from any unreacted gramicidin (R_F value about 75). As an alternative solvent system we used butanol/acetic acid/water, 63:27:10 (v/v), with an R_F value of about 67 for O-pyromellitylgramicidin.

The following analytical tests were carried out with the final product. Alkaline hydrolysis in methanol/water 1:1 (pH 10–11, 20 °C, 5 hr) yielded a compound which was indistinguishable on a thin-layer chromatogram from gramicidin A. The (UV) spectrum of the saponification product showed the typical absorption of tryptophan at 280 nm with a molar extinction coefficient of $\varepsilon = 19{,}600$ counted for a molecular weight of 1860. ε of gramicidin was 20,500. These data indicate that no reaction of tryptophan with pyromelitic acid anhydride occured. The infrared spectrum of O-pyromellitylgramicidin was identical with the spectrum of gramicidin A, except for an additional ester-carbonyl peak at 5.8 μm. The spectra were recorded from KBr discs in a Perkin-Elmer spectrophotometer Model 621. The homogeneity and the molecular weight was checked by sedimentation analysis. A 9.4 nM solution in water was centrifugated in a Beckman analytical ultracentrifuge type E (An-D rotor with a 12 mm double sector cell) for three days at 6×10^5 rpm. The logarithmic plot of concentration *vs.* the square of the radial distance was linear, indicating that the material was homogeneous. Using a value of 0.75 $cm^3 g^{-1}$ for the partial specific volume of peptides in water, the molecular weight was calculated to be 2010 g/mole (theoretical value: 2086 g/mole). The pK values of pyromellitic acid are $pK_1 = 1.92$, $pK_2 = 2.89$, $pK_3 = 4.49$, $pK_4 = 5.64$ (Maxwell & Partington, 1937). From a methanol/water solution (1:1) the free acid is precipitated by acidification. The UV-spectrum of the O-pyromellitylgramicidin in the free acid form had a maximum at 270 nm with a molar extinction coefficient of $\varepsilon = 21{,}700$ counted for a molecular weight of 2086. After saponification the resulting gramicidin shows the same UV spectrum for tryptophan residues as the normal gramicidin. The free acid dissolves in methanolic–aqueous $NaHCO_3$, producing CO_2. 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine (dioleoyllecithin) was synthetized by K. Janko (Benz, Stark, Janko & Läuger, 1973). The monoglycerides with C_{18}, C_{22} and C_{24} cis-monounsaturated fatty acids (monoolein, monoerucin, mononervonin) were obtained from Nu-Check Preparation, Elysian, Minnesota. The samples consisted mainly of the α isomers with small amounts of the β isomers. *n*-decane and *n*-hexadecane were from Merck, Darmstadt (standards for gas chromatography). All other reagents were analytical grade. Black lipid membranes were formed in the usual way (Läuger, Lesslauer, Marti & Richter, 1967) in a thermostated teflon cell filled with an aqueous electrolyte solution. The lipid was dissolved in *n*-decane or *n*-hexadecane (1–2.5%, w/v). Different types of teflon cells were used with varying diameters of the hole in the septum. The membrane area was determined with an eyepiece micrometer and was between 8×10^{-3} and 7×10^{-2} cm^2 for the macroscopic conductance measurements and about 3×10^{-4} cm^2 for the single-channel measurements. O-pyromellitylgramicidin was added to the aqueous phase from a methanolic stock solution. Relaxation experiments (Bamberg & Läuger, 1973) and single-channel measurements (Bamberg & Läuger, 1974) were carried out as described previously.

Results

Steady-state Conductance

If O-pyromellitylgramicidin is added to one side of a lipid membrane in the presence of 0.2 M CsCl only a small membrane conductance de-

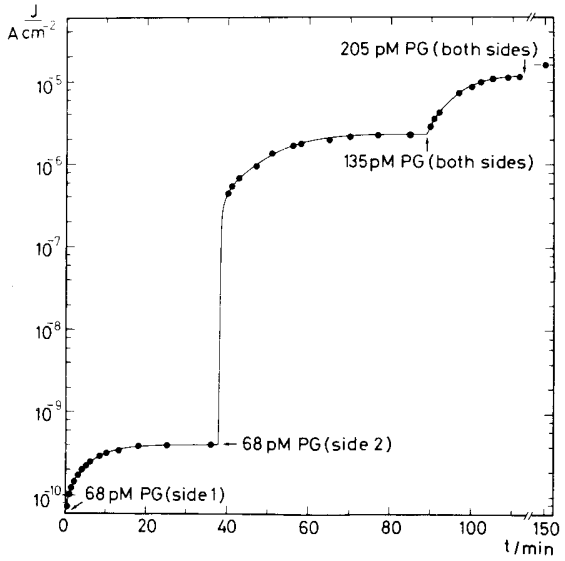

Fig. 2. Membrane current J after asymmetric and symmetric addition of O-pyromellitylgramicidin (PG). The membrane was formed from dioleoyllecithin in n-decane. Both aqueous solutions contained 0.2 M CsCl, pH $\simeq$ 6.0 (unbuffered), $T = 25$ °C. The applied voltage was 25 mV. At time $t = 0$, 5 µl of a 2×10^{-7} M methanolic solution of PG were added to one aqueous compartment to give a concentration of 6.8×10^{-11} M. At $t = 38$ min the same amount of PG was added to the other compartment; additional amounts were symmetrically added later to both sides up to a final concentration of 2.05×10^{-10} M

velops (Fig. 2). If the compound is then added also to the other side, a drastic increase of conductance is observed; under the experimental conditions given in Fig. 2 the conductance changes by a factor of about 5×10^3. This finding is in sharp contrast to the behavior observed with unmodified gramicidin which yields nearly the same membrane conductance, irrespective of whether the peptide is added to only one or to both aqueous compartments. In fact, the marginal conductance which develops after asymmetric addition of O-pyromellitylgramicidin probably results from traces of gramicidin present in the sample.

These findings can be explained on the basis of the dimer model if it is assumed that the two halves of the channel can combine only if they are present on opposite sides of the bilayer. Unmodified gramicidin which is strongly hydrophobic is likely to easily cross the membrane, whereas the negatively charged O-pyromellitylgramicidin presumably stays on the side of the bilayer where it has been added. With gramicidin it has proved to be rather difficult to determine the dependence of membrane conductance λ on the aqueous concentration of the peptide (Toste-

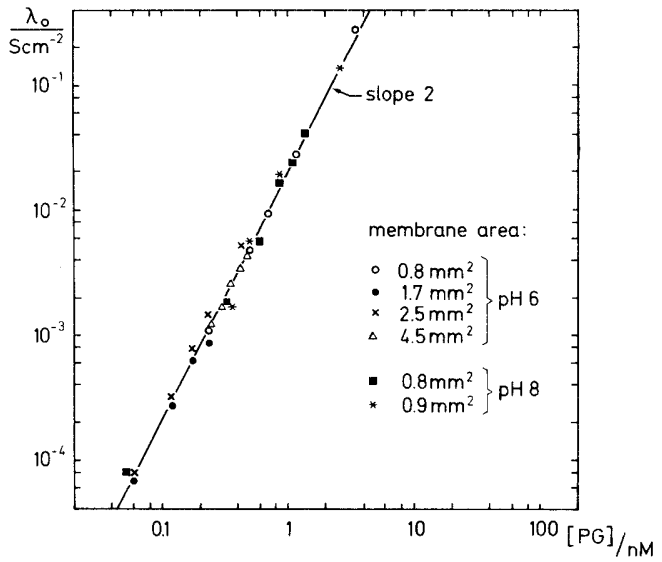

Fig. 3. Membrane conductance λ_o, expressed in S cm^{-2} (1 S = 1 Ω^{-1}), as a function of the concentration [PG] of O-pyromellitylgramicidin in water. Dioleoyllecithin/*n*-decane membrane, 0.2 M CsCl, T = 25 °C. The applied voltage was 25 mV. The values of λ_o were obtained from different membranes with areas between 0.8 and 4.5 mm^2. In each experiment the PG concentration was varied by successive symmetrical additions to both aqueous solutions. The pH of the solutions was either ~6 (unbuffered) or 8.0 (0.1 mM Tris)

son, Andreoli, Tieffenberg & Cook, 1968; Hladky & Haydon, 1972; Veatch *et al.*, 1976). The difficulty in obtaining reproducible values of λ with given amounts of gramicidin added to the aqueous solutions apparently results from the strongly hydrophobic nature and the poor water solubility of gramicidin. On the other hand, with O-pyromellitylgramicidin which has a much higher water-solubility, reproducible conductance measurements can easily be performed. Results of such experiments are represented in Fig. 3. Most of these experiments were done in unbuffered solutions at a pH of about 6. A few control experiments were carried out at pH 8.0 (0.1 mM Tris) which gave results almost identical with those at pH 6, indicating that the ionization state of the pyromellityl residue does not appreciably change between pH 6 and 8. It is seen from Fig. 3 that the membrane conductance λ_0 is proportional to the square of the aqueous concentration of O-pyromellitylgramicidin over a concentration range of at least 1:60. This result, of course, has to be expected if the channel consists of a dimer.

Relaxation Experiments

Previous experiments have shown that after a sudden change of the applied voltage the current through a gramicidin-doped membrane increases exponentially towards a new stationary value (Bamberg & Läuger, 1973; Bamberg & Benz, 1976). On the basis of the dimer model, this current relaxation is explained as a shift in the monomer-dimer equilibrium in the membrane, which is induced by a change in the electric field strength. If k_R and k_D are the rate constants of association and dissociation of the gramicidin dimer G_2:

$$G + G \underset{k_D}{\overset{k_R}{\rightleftharpoons}} G_2 \tag{1}$$

and if Λ is the conductance of the single channel, the relaxation time τ of the membrane current is given by (Bamberg & Läuger, 1973):

$$\frac{1}{\tau} = k_D + 4\sqrt{\frac{k_R k_D \lambda_\infty}{L\Lambda}} \tag{2}$$

λ_∞ is the stationary membrane conductance at infinite time after the voltage jump and L is Avogadro's number

Voltage-jump relaxation experiments with O-pyromellitylgramicidin gave results which were qualitatively similar to those obtained with gramicidin (Bamberg & Läuger, 1973). The increase of membrane current after the voltage-jump was exponential and could be described by a single relaxation time τ. By varying the gramicidin concentration in water, τ was measured at different values of the stationary membrane conductance λ_∞. In Fig. 4 the observed values of $1/\tau$ are plotted *vs.* the square root of λ_∞; it is seen that the experimental points fall near a straight line; such a linear relationship between $1/\tau$ and $\sqrt{\lambda_\infty}$ is expected from Eq. 2. From the slope of the straight line and the intercept with the $1/\tau$ axis the values of k_D and k_R may be obtained. Using a value of $\Lambda = 10$ pS which has been separately determined from single-channel experiments (*see* below) the result reads: $k_D = 1.4\,\text{sec}^{-1}$, $k_R = 4 \times 10^{13}\,\text{cm}^2\,\text{mol}^{-1}\,\text{sec}^{-1}$. These values are similar to the rate constants determined for gramicidin under otherwise identical conditions: $k_D = 1.6\ \text{sec}^{-1}$, $k_R = 2.4 \times 10^{14}\ \text{cm}^2\ \text{mol}^{-1}\ \text{sec}^{-1}$ (Bamberg & Läuger, 1973). This means the presence of the pyromellityl residue does not drastically change the kinetics of channel formation.

From the equilibrium constant of dimerization, $K = k_R/k_D$, and from the relationship between the specific membrane conductance λ and the

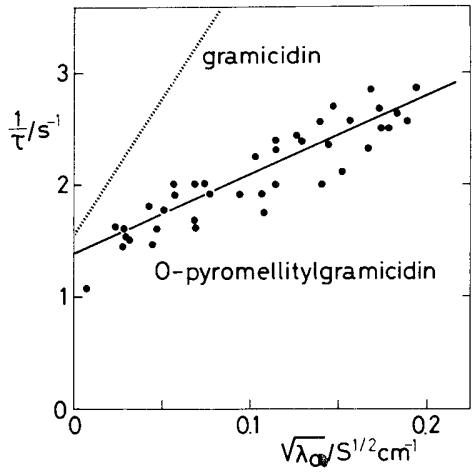

Fig. 4. Analysis of voltage-jump relaxation experiments with O-pyromellitylgramicidin according to Eq. 2. Reciprocal value of the relaxation time τ plotted as a function of the square root of the stationary membrane conductance. Dioleoyllecithin/*n*-decane membrane, 1 M NaCl, pH $\simeq$ 5.9 (unbuffered), 25 °C. The amplitude of the voltage jump was 135 mV. For comparison, the result from previous studies with gramicidin (Bamberg & Läuger, 1973) is also given

concentration c of O-pyromellitylgramicidin in water (Fig. 3) the partition coefficient $\beta = N_m/c$ between membrane and water may be determined (N_m is the interfacial concentration of the monomer in one half of the membrane, expressed in mole/cm^2). Replacing N_m by the dimer concentration N_d according to $K = N_d/N_m^2$ and using the relation $\lambda = LN_d\Lambda$, the partition coefficient is obtained as

$$\beta = \frac{1}{c}\sqrt{\frac{\lambda}{KL\Lambda}}. \tag{3}$$

With $\sqrt{\lambda/c} = 1.4 \times 10^{11}$ S$^{1/2}$ cm^2 mole^{-1} (0.2 M CsCl, Fig. 3), $\Lambda = 50$ pS (0.2 M CsCl, *see* below), $K = k_R/k_D = 2.9 \times 10^{13}$ cm^2 mole^{-1}, β is calculated to be 4.8×10^{-3} cm. Sometimes the partition coefficient is defined as a dimensionless quantity $\gamma = 2\ N_m/cd$, where $d \sim 5$ nm is the membrane thickness. From the value of β given above, $\gamma = 1.9 \times 10^4$ is obtained.

The voltage dependence of the membrane conductance is represented in Fig. 5, both for the initial state immediately after the voltage jump ($t = 0$) and for the stationary state ($t \to \infty$). It is seen that the initial conductance is independent of voltage up to at least 200 mV at 0.1 M NaCl, and only slightly voltage dependent at 1 M NaCl. This is in agreement with the data obtained by Hladky & Haydon (1972), who found

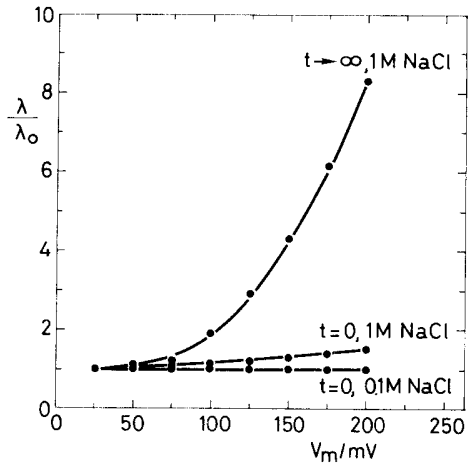

Fig. 5. Voltage dependence of membrane conductance λ in the initial state, immediately after the voltage jump ($t=0$), and in the stationary state ($t\to\infty$). The conductance has been normalized by dividing through the ohmic conductance λ_o measured at 25 mV. $\lambda(t=0)$ has been obtained by extrapolating the time course $J(t)$ of the current in the relaxation experiment to time zero. Dioleoyllecithin/*n*-decane membrane, $T=25$ °C. The aqueous phases contained either 0.1 or 1 M NaCl and various concentrations of O-pyromellitylgramicidin (between 10^{-11} and 10^{-10} M); the pH was about 6 (unbuffered solutions)

that at very high concentration (> 1 M) the current voltage curve shows a small superlinear behavior. That means in case of the O-pyromellitylgramicidin the ion concentration on the mouth of the pore is greater than 1 M because of the negative charges. The stationary conductance, on the other hand, shows a strong increase with voltage which reflects the enhanced probability of channel formation at higher field strengths.

Single-channel Analysis

At very low concentrations of O-pyromellitylgramicidin the membrane conductance fluctuates in a stepwise fashion (Fig. 6). This behavior is similar to that observed with gramicidin, but the two compounds differ in the size of the conductance steps and slightly in the mean duration. A histogram of the conductance fluctuations in 0.2 M CsCl solution is shown in Fig. 7. The sample of O-pyromellitylgramicidin used in the experiment represented in Fig. 7 contained a small amount of gramicidin. (Trace amounts of unmodified gramicidin were often detectable in preparations of O-pyromellitylgramicidin and could only be removed by careful purification.) Accordingly, the probability distribu-

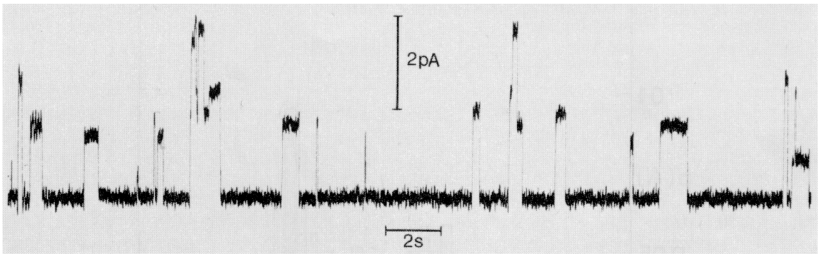

Fig. 6. Current fluctuations at low concentration of O-pyromellitylgramicidin. Monoolein/*n*-hexadecane membrane, $T=25$ °C, 0.01 M CsCl. The applied voltage was 100 mV

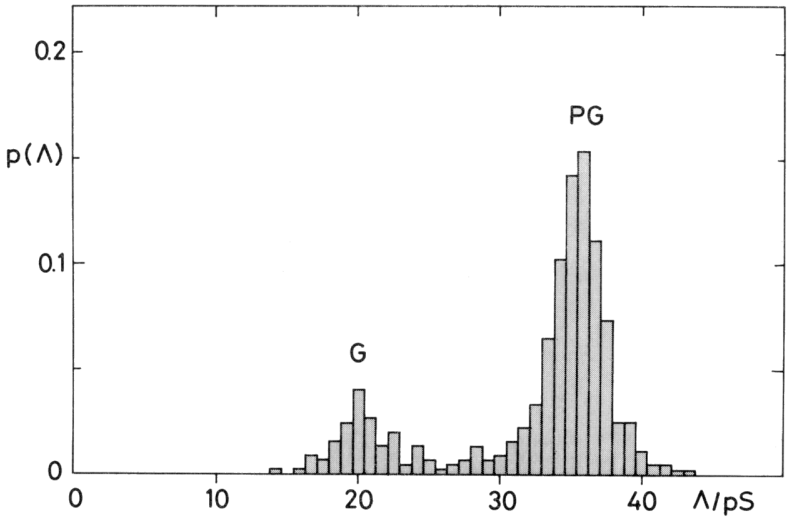

Fig. 7. Probability $p(\Lambda)$ of occurrence of a conductance fluctation of magnitude Λ. $p(\Lambda)$ is the number of events within an interval of width $\Delta\Lambda = \pm 0.42$ pS centered at Λ, divided by the total number n=500 of events. The sample of O-pyromellitylgramicidin A(PG) contained a trace amount of gramicidin A(G). The membrane was formed from monoerucin [(22:1)-monoglyceride] in *n*-hexadecane. 0.2 M CsCl, pH≃6 (unbuffered), $T=25$ °C

tion $p(\Lambda)$ of the single-channel conductance Λ shows two peaks, one at $\Lambda \simeq 20$ pS which corresponds to the most probable single-channel conductance of pure gramicidin in 0.2 M CsCl, and a second peak at 35 pS which is attributed to O-pyromellitylgramicidin. The pH dependence of $p(\Lambda)$ was checked by carrying out the analysis at pH ~6 (unbuffered solutions) and at pH 8.0 (0.1 mM Tris). The magnitude of the most probable Λ for O-pyromellitylgramicidin differed by only 6% at the two pH values.

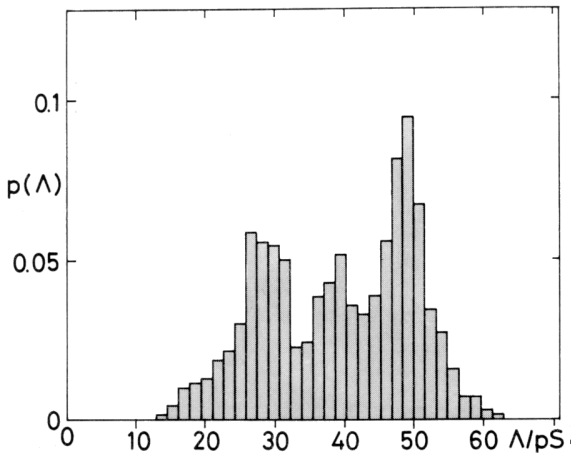

Fig. 8. Probability $p(\Lambda)$ of conductance fluctuations in the presence of a mixture of gramicidin A and O-pyromellitylgramicidin A. Monoolein/*n*-hexadecane membrane in 0.2 M CsCl, pH $\simeq$ 6 (unbuffered), $T = 25$ °C. The total number of events was n = 700

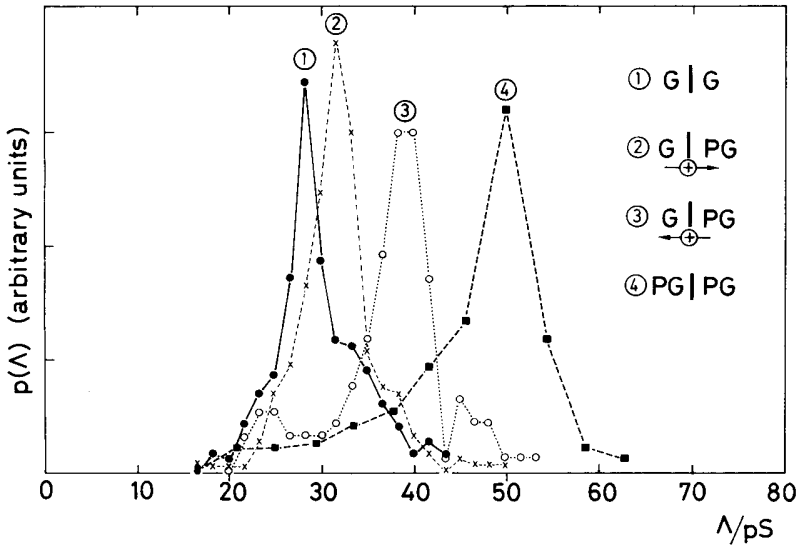

Fig. 9. Probability $p(\Lambda)$ of conductance fluctuations (in arbitrary units) after symmetric and asymmetric additions of gramicidin A(G) and O-pyromellitylgramicidin A(PG) to the aqueous solutions. Monoolein/*n*-hexadecane membrane, 0.2 M CsCl, $T = 25$ °C. Gramicidin A was added to give a nominal concentration of $\sim 10^{-14}$ M and O-pyromellitylgramicidin A to give a concentration of $\sim 10^{-11}$ M. The applied voltage was ± 50 mV. In the experiment represented by curve 2 the polarity of the voltage was such that positive ions passed through the membrane from the gramicidin side to the O-pyromellitylgramicidin side; curve 3 was obtained with the reverse polarity. In the symmetrical systems (curves 1 and 4) the conductance was independent of polarity. For each system the values of $p(\Lambda)$ have been multiplied by an arbitrary scale factor in order to obtain comparable peak heights

If the ratio of gramicidin to O-pyromellitylgramicidin is such that both peaks in the probability distribution become of comparable height, a third, smaller peak appears between the two main peaks (Fig. 8). A likely explanation of this intermediate peak is that it results from hybrid pores which are formed by association of a gramicidin monomer with a O-pyromellitylgramicidin monomer. Such hybrid pores have also been observed by W. Veatch (1976) and Veatch and Stryer (1976) with a chemically modified gramicidin C. The finding that a mixture of two different gramicidin species yields, in addition to channels corresponding to the pure compounds, a single probability peak at intermediate conductances further supports the dimer model of the gramicidin channel.

If the channels of intermediate conductance are indeed hybrid dimers composed of a neutral and a negatively charged gramicidin monomer, then the current-voltage characteristic of the individual hybrid channel should be asymmetric. This expectation has been tested in experiments in which a small amount of gramicidin was added to side 1 and a much larger amount of O-pyromellitylgramicidin to side 2 (Fig. 9, curves 2 and 3). Under these circumstances the predominant form of the channel should be a hybrid with the neutral monomer pointing to side 1 and the negatively charged monomer pointing to side 2. It was found that, if cations were driven through the channel from the neutral side (side 1) to the negative side (side 2), the conductance was smaller by about 20% than for the opposite polarity (Fig. 9). Thus, even at the relatively low voltages applied in these experiments (± 50 mV) the single channel exhibits a distinct asymmetry in the current-voltage characteristic. The sign of the asymmetry is in accordance with expectation for a cation-permeable channel.

The dependence of single-channel conductance Λ on ion concentration is shown in Fig. 10. At high concentration the single-channel conductance of O-pyromellitylgramicidin is smaller than that of gramicidin. This difference possibly arises from the partial obstruction of the channel mouth by the bulky pyromellityl residue. At low ion concentrations, however, the O-pyromellitylgramicidin channel has a considerably higher conductance than the neutral channel (*see* also Fig. 11). This finding is indeed what has to be expected from the high density of negative charge at the mouth of the O-pyromellitylgramicidin channel. The electrical potential created by the fixed negative charges leads to an accumulation of permeable positive ions near the opening of the channel; according to the Debye-Hückel theory of interionic attraction, this effect should become stronger at low ionic strength (*compare* Fig. 11).

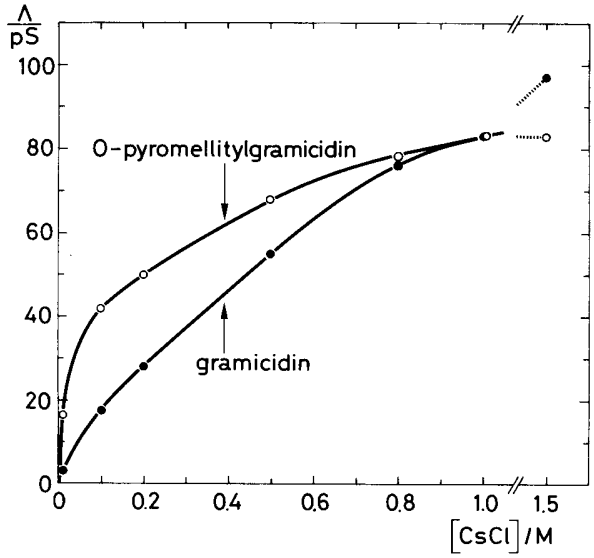

Fig. 10. Dependence of single-channel conductance Λ on the concentration of CsCl in the aqueous phase. Monoolein/*n*-hexadecane membrane, $T=25$ °C. The observed values of Λ for gramicidin closely agree with those reported by Hladky and Haydon (1972)

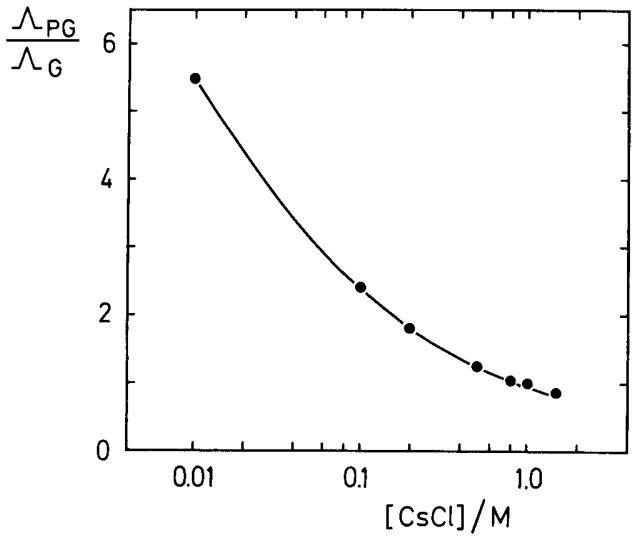

Fig. 11. Single-channel conductance Λ_{PG} of O-pyromellitylgramicidin divided by the single-channel conductance Λ_G of gramicidin. Data of Fig. 10

Table 1. Channel mean lifetime τ^* (in sec) of O-pyromellitylgramicidin in membranes made from three different monoglycerides: monoolein [(18:1)-MG], monoerucin [(22:1)-MG], and mononervonin [(24:1)-MG][a]

	(18:1)-MG *n*-decane	(18:1)-MG *n*-hexadecane	(22:1)-MG *n*-hexadecane	(24:1)-MG *n*-hexadecane
O-pyromellitylgramicidin	0.46	2.2	0.065	0.046
gramicidin	0.24	2.0	0.043	~0.010

[a] The solvent was either *n*-decane or *n*-hexadecane. The aqueous phase contained 1 M CsCl, the temperature was 25 °C in the case of (18:1)-MG and (22:1)-MG, and 27 °C in the case of (24:1)-MG. For comparison, the values of τ^* for gramicidin are also given (Bamberg *et al.*, 1975; Kolb & Bamberg, 1976).

The mean lifetime τ^* of the channel was determined from the records of the current fluctuations (Fig. 6) by plotting the logarithm of the number of fluctuations with a lifetime greater than t *vs.* t. From the slope of the resulting straight line the value of τ^* was obtained. The results are given in Table 1. It is seen that, depending on the composition of the membrane, the lifetime of the O-pyromellitylgramicidin channel is larger by a factor between 1.1 and 4.6 than the lifetime of the gramicidin channel.

Discussion

The experiments with the negatively charged pyromellityl derivative of gramicidin described above indicate that this compound, like gramicidin itself, forms ion-permeable channels in lipid bilayer membranes. The large amplitude of the conductance fluctuations observed at very low concentrations of the negatively charged analog, which is of the same order of magnitude or larger than the fluctuation amplitude observed with gramicidin, makes a carrier model rather unlikely and supports the notion of a channel mechanism (Hladky & Haydon, 1972). Furthermore, the experiments give evidence that the channel is formed by association of two monomers: first, the macroscopic membrane conductance is found to be a quadratic function of aqueous O-pyromellitylgramicidin concentration (Fig. 3). This is consistent with the dimer hypothesis if it is assumed that the concentration of the monomer in the membrane is proportional to the concentration in water over the whole concentration range. Second, the observation that the membrane conductance remains

low if O-pyromellitylgramicidin is added to only one side but increases by several orders of magnitude if the same amount is also added to the other side (Fig. 2) is easily explained on the basis of the dimer model, but would be difficult to understand if the conducting unit would be a monomer. Third, the occurrence of channels of intermediate conductance in mixtures of gramicidin and O-pyromellitylgramicidin (Fig. 8) is also consistent with the dimerization hypothesis.

The experiments described here restrict the number of possible channel structures. In the original model proposed by Urry (1971, 1972) the channel consists of a dimer which is formed by head-to-head (formyl end to formyl end) association of two gramicidin monomers. Urry, and later Veatch, Fossel and Blout (1974) also discussed the possibility of head-to-tail and tail-to-tail dimers. Besides this, Veatch *et al.* introduced a new class of double helices in which the two peptides are coiled about a common axis. These double-stranded helices may occur in a form with parallel and in an alternative form with antiparallel orientation of the two peptide chains. Our experiments may be interpreted on the basis of either the head-to-head dimer of Urry or the antiparallel double-stranded helix of Veatch *et al.* From the experiment with asymmetric addition of O-pyromellitylgramicidin we can exclude the double-stranded helix with parallel orientation of the peptide chains. Also, in view of the high amount of energy required to transfer the charged pyromellityl residue into the apolar interior of the membrane, the head-to-tail and tail-to-tail structures seem extremely unlikely. These arguments, of course, hold for the negatively charged derivative; in view of the similarity between its single-channel properties and those of gramicidin, the conclusions drawn above may also be applied (with some caution) to gramicidin itself.

The pyromellityl residue introduces negative charges at the mouth of the channel. The pK values of pyromellitic acid in water are $pK_1 = 1.92$, $pK_2 = 2.89$, $pK_3 = 4.49$, $pK_4 = 5.64$ (Maxwell & Partington, 1937); in the case of the monoester of pyromellitic acid the pK values are likely to lie in the same range. The pK values of membrane-bound O-pyromellitylgramicidin are not known, however. It is therefore questionable whether all three carboxyl groups are dissociated at $pH \simeq 6$ where most of our experiments have been carried out. But from the finding that neither the macroscopic conductance nor the single-channel conductance did appreciably change when the pH was increased from 6 to 8, it seems likely that all carboxyl groups of the membrane-bound molecule are ionized. The effect of the pyromellityl residue on the conductance

of the channel may be twofold: the bulky residue may partially block the entrance of the channel; on the other hand, the negative potential created by the ionized carboxyl groups should increase the concentration of permeable cations near the channel mouth and therefore should enhance the conductance. The observation that at high ion concentrations (where the coulombic effect is small) the conductance of the O-pyromellitylgramicidin channel is lower than that of the gramicidin channel may be tentatively explained by a steric blocking effect. The electrostatic effect becomes evident at low ion concentrations c where the conductance of the negative channel is enhanced 5.6-fold (at $c = 10^{-2}$ M) over that of the neutral channel (Fig. 11). A very rough estimate (to the order of magnitude) of the expected electrostatic modification of the single-channel conductance may be made in the following way. We represent the pyromellityl residue by a charged sphere of radius a centered at the point where the channel axis intersects the membrane surface. The electrical potential at the surface of the sphere is then approximately given by the Debye-Hückel relation:

$$\varphi(a) = \frac{z e_0^2}{4\pi\varepsilon_0 \varepsilon a k T} \cdot \frac{1}{1 + a/l_D} \simeq \frac{z(56.0\text{ nm})}{\varepsilon a} \cdot \frac{1}{1 + a/l_D}; \tag{4}$$

$$l_D = \sqrt{\frac{\varepsilon_0 \varepsilon k T}{2 e_0^2 c}} \quad (25\,^\circ\text{C}). \tag{5}$$

e_o is the elementary charge, z the valency, ε_o the permitivity of free space, ε the dielectric constant, k Boltzmann's constant, T the absolute temperature, c the concentration of the 1:1 electrolyte in water, and l_D the Debye length; the potential φ is expressed in units of kT/e_o. For a more detailed discussion of the effects of discrete charges in the membrane-solution interface, *see*, for instance, Cole (1969), Nelson and McQuarrie (1975), and Brown (1975). If the single-channel conductance Λ is assumed to be proportional to the ion concentration at the mouth of the channel, then exp (φ) appears as a Boltzmann factor in the expression for Λ. The channel mouth may be represented by a hemispherical surface with radius r_o, the so-called effective capture radius (Läuger, 1976). If, for the purpose of a crude estimate, we identify the capture radius of the channel with the radius a, the ratio q of the conductance Λ_c of the charged channel to the conductance Λ_u of the uncharged channel in the same electrolyte solutions is given by

$$q = \frac{\Lambda_c}{\Lambda_u} \simeq e^{-\varphi(a)}. \tag{6}$$

In order to eliminate the steric blocking effect of the pyromellityl residue, it is convenient to consider ratios of q values determined at two different ion concentrations c_1 and c_2. The experimental results for $q(c_1)/q(c_2)$ may indeed be fitted with reasonable values of a and ε. For instance, $\varepsilon = 80$, $a = 0.6$ nm, and $z = -3$ gives $q(0.01\text{ M})/q(1\text{ M}) = 5.7$ and $q(0.1\text{ M})/q(1\text{ M}) = 2.7$ which is rather close to the experimentally observed values (Fig. 11). A more exact treatment should account for the precise location of the fixed charges with respect to the channel, the discontinuity of the dielectric constant at the membrane surface, deviations from the Debye-Hückel theory at high concentration, and saturation effects in the channel. In any case, the observed variation of Λ_{PG}/Λ_G with ion concentration at least qualitatively agrees with expected effects of fixed charges on the conductance of the channel.

At high channel densities in the membrane the possibility of an overlap of the ion atmospheres surrounding the fixed charges and a concomitant channel-channel interaction has to be envisaged. Under the conditions of our experiments this effect is quite negligible, however. For instance, if the membrane conductance in 0.2 M CsCl is 10^{-2} S cm^{-2} (Fig. 3), the density of channels (conductance $\Lambda \simeq 50$ pS) is 2×10^8 cm^{-2}. This corresponds to a mean distance of 700 nm which is large compared with the Debye length $l_D \simeq 0.68$ nm in 0.2 M CsCl.

A further manifestation of the electrostatic effect of fixed charges consists in the asymmetry of the current-voltage characteristic of hybrid channels (Fig. 9). This rectification results from the fact that if a positive potential is applied to the aqueous solution facing the negative end of the channel, permeable cations enter the channel from the side where their concentration is increased by the presence of the fixed charges. The simplest way to analyze the current-voltage characteristic of the asymmetric channel consists in treating the passage of the ion through the channel as the migration in a quasi-continuum of many identical energy barriers. If the charged end of the channel is assumed to face the left-hand solution, and if φ_o is the electrical potential at the left channel mouth with respect to the solution, the continuum model of the channel yields the following expression for the rectification ratio φ (Läuger, *unpublished*):

$$\rho \equiv \frac{J(u)}{-J(-u)} = \frac{\varphi_0 + u}{\varphi_0 - u} e^{-u} \frac{e^{\varphi_0} - e^{u}}{e^{\varphi_0} - e^{-u}} . \tag{7}$$

J is the current through the channel and $u = \varphi' - \varphi''$ the voltage between the left-hand ($'$) and the righthand ($''$) aqueous solution; φ_o and u are

expressed in units of $kT/e_o \simeq 25.6$ mV (25 °C). The agreement between the observed value of φ and the value predicted from Eq. 7 is poor, however. The experiment represented in Fig. 9 (0.2 M CsCl, $u=1.95$) yields $\varphi \simeq 1.22$. On the other hand, $\varphi_o \simeq \varphi(a)$ max be estimated from Eq. 4 by using $\varepsilon=80$, $z=-3$, $l_D=0.68$ nm (0.2 M CsCl) and the value of a which gave the best fit for Λ_{PG}/Λ_G ($a=0.6$ nm); this yields $\varphi_o=-1.86$ and (using Eq. 7) $\varphi=1.72$. The discrepancy between the calculated and the observed value of φ presumably originates from the use of a continuum approximation for the channel. A more realisitic treatment should become possible as soon as more information about the energy profile of the channel is available.

The authors wish to thank U. Markau for carrying out the sedimentation analysis, Dr. K. Markau and Dr. J. Rasched for helpful discussions, and B. Neubert for excellent technical assistance. This work has been financially supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 138).

References

Bamberg, E., Benz, R. 1976. Voltage-induced thickness changes of lipid bilayer membranes and the effect of an electric field on gramicidin A channel formulation. *Biochim. Biophys. Acta* **426**:570

Bamberg, E., Kolb, H.-A., Läuger, P. 1976. Ion transport through the gramicidin A channel. *In:* The Structural Basis of Membrane Function. Y. Hatefi and L. Djavadi-Ohaniance, editors. Academic Press, New York

Bamberg, E., Läuger, P. 1973. Channel formation kinetics of gramicidin A in lipid bilayer membranes. *J. Membrane Biol.* **11**:177

Bamberg, E., Läuger, P. 1974. Temperature-dependent properties of gramicidin A channels. *Biochim. Biophys. Acta* **367**:127

Bamberg, E., Noda, K., Gross, E., Läuger, P. 1975. Single-channel parameters of gramicidin A, B and C. *Biochim. Biophys. Acta* **419**:223

Benz, R., Stark, G., Janko, K., Läuger, P. 1973. Valinomycin-mediated ion transport through neutral lipid membranes: Influence of hydrocarbon chain length and temperature. *J. Membrane Biol.* **14**:339

Brown, R.H., Jr. 1974. Membrane surface charge: Discrete and uniform modelling. *Progr. Biophys. Mol. Biol.* **28**:341

Cole, K.S. 1969. Zeta potential and discrete *vs.* uniform charges. *Biophys. J.* **9**:465

Drouin, H., Neumcke, B. 1974. Specific and unspecific charges at the sodium channels of the nerve membrane. *Pfluegers Arch.* **351**:207

Hille, B., Woodhull, A.M., Shapiro, B.I. 1975. Negative surface charge near sodium channels of nerve: Divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. B* **270**:301

Hladky, S.B., Haydon, D.A. 1970. Discreteness of conductance changes in bimolecular lipid membranes in the presence of certain antibiotics. *Nature (London)* **225**:451

Hladky, S.B., Haydon, D.A. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochim. Biophys. Acta* **274**:294

Kolb, H.-A., Bamberg, E. 1976. Influence of Membrane Thickness and ion concentration on the properties of the gramicidin A channel: Autocorrelation, spectral power density, relaxation and single channel studies. *Biochim. Biophys. Acta* (*in press*)

Kolb, H.-A., Läuger, P., Bamberg, E. 1975. Correlation analysis of electrical noise in lipid bilayer membranes: Kinetics of gramicidin A channels. *J. Membrane Biol.* **20:**133

Läuger, P. 1976. Diffusion-limited ion flow through pores. *Biochim. Biophys. Acta* (*in press*)

Läuger, P., Lesslauer, W., Marti, E., Richter, J. 1967. Electrical properties of bimolecular phospholipid membranes. *Biochim. Biophys. Acta* **135:**20

Maxwell, W.R., Partington, J.R. 1937. The dissociation constants of some polybasic acids. Part III. *Trans. Faraday Soc.* **33:**670

Nelson, A.P., McQuarrie, D.A. 1975. The effect of discrete charges on the electrical properties of a membrane. *J. Theor. Biol.* **55:**13

Ovchinnikov, Yu.A. 1972. Structure and membrane activity of peptide ionophores. *In:* Federation of European Biochemical Societies, 8th Meeting, Vol. 28, p. 279*ff.* North Holland, Amsterdam

Tosteson, D.C., Andreoli, T.E., Tieffenberg, M., Cook, P. 1968. The effects of macrocyclic compounds on cation transport in sheep red cells and thin and thick lipid membranes. *J. Gen. Physiol.* **51:**373*S*

Urry, D.W. 1971. The gramicidin A transmembrane channel: A proposed $\pi_{(L,D)}$ helix. *Proc. Nat. Acad. Sci.* **68:**672

Urry, D.W. 1972. A molecular theory of ion-conducting channels: A field-dependent transition between conducting and nonconducting conformations. *Proc. Nat. Acad. Sci.* **69:**1610

Veatch, W.R. 1976. The structure of the Gramicidin A trans-membrane Channel *20th Annu. Meet. Biophys. Soc.* (Abstr.)

Veatch, W.R., Fossel, E.T., Blout, E.R. 1974. The conformation of gramicidin A. Biochemistry **13:**5249

Veatch, W.R., Mathies, R., Eisenberg, M., Stryer, L. 1976. Simultaneous fluorescence and conduction studies of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A. J. Mol. Biol. **99:**75

Veatch, W.R., Stryer, L. 1976. The dimeric nature of the gramicidin A transmembrane channel, conductance and fluorescence energy transfer studies of hybrid channels. *J. Mol. Biol.* (*in press*)

Woodhull, A.M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61:**687

Zingsheim, H.P., Neher, E. 1974. The equivalence of fluctuation analysis and chemical relaxation experiments: A kinetic study of ion pore formation in thin lipid membranes. *Biophys. Chem.* **2:**197