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Impedance analysis of phosphatidylcholine membranes modified with gramicidin D

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Abstract

Electrochemical impedance spectroscopy (EIS) was used to the study of gramicidin D (GD) dimerization and to transport of monovalent cations across lipid bilayers by the dimers. Phosphatidylcholine (PC) membranes were studied, unmodified and modified with very low GD concentrations in the presence of various potassium ion concentrations. A new method was proposed to determine the parameters used to describe the gramicidin dimer: gramicidin surface concentration (c_{GG}), area occupied by individual channel (A_G) and gramicidin dimerization equilibrium constant (K_{GG}). It was shown that electrochemical impedance spectroscopy measurements of lipid bilayer membranes yielded the K_{GG} and A_G values of the same order of magnitude as other measurement techniques.

Keywords: BLM; EIS; Phosphatidylcholine; Gramicidin D dimers

1. Introduction

An important aim of biophysicists is understanding of mechanisms making biological ionic channels be efficient in ion transport control. Detailed study of individual membrane functions, e.g., of ion transport, is facilitated by simply building artificial lipid bilayers, whereas biological membranes are complex structures of lipids and proteins. Transmembrane peptides have been often used as models of membrane proteins and channels because they can be more readily characterized and introduced into the bilayer. Gramicidin is a model of more complex biological ionic channels. For this reason, many studies have been done using this simple channel-forming polypeptide.

Linear gramicidin A, B and C are able to induce ion transfer across both natural and artificial lipid membranes [1–3]. These pentadecapeptides are built of L- and D-amino acids arranged alternately in chain, their terminal amino group is formylated and ethanolamine is substituted to their terminal carboxy group. Gramicidin A, B and C differ with only one amino acid: the tryptophan residue appearing at position 11 in gramicidin A is replaced by the L-phenylalanine residue in gramicidin B and by the L-tyrosine residue in gramicidin C. Gramicidin D (GD) is a natural mixture of 85% of gramicidin A, 10% of gramicidin C and 5% of gramicidin B [4].

The GD monomers introduced into the bilayer can form formyl-NH to formyl-NH (N-to-N) dimeric species. The dimers stretch across the bilayer and they can constitute ionic channels, which are able to transport monovalent cations. In the N-to-N dimer model, the monomers are connected by 12 intermolecular hydrogen bonds and the dimer is stabilized by six intermolecular hydrogen bonds linking the formyl groups and the N-terminal amino acids mutually [5,6]. It is suggested that opening and closing of the channels is connected with the association of the intermolecular hydrogen bonds. The dimer-building monomers separate if their bonds are broken hereby destroying the transmembrane channel. At low concentration, gramicidin in the lipid bilayer can exist both as monomers and as dimers [7]; at relatively high concentrations it appears as dimers only [6].

Useful information on gramicidin channel formation kinetics and on conductance of individual channel was obtained from various experimental methods [8–10]. In this paper, application of electrochemical impedance spec-

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troscopy (EIS) to the study of electrochemical behaviour of a channel introduced into a lipid bilayer is described. Modification of a phosphatidylcholine (PC) membrane with gramicidin, recording variations in the impedance spectra caused by the modification and determination of characteristic parameters of gramicidin dimer in terms of suitable electric models have been the aim of this work.

2. Experimental

2.1. Materials

Gramicidin D was purchased from Sigma (St. Louis, MO) and used without further purification. Egg PC (99%) was from Fluka (Neu-Ulm, Germany) and it had the following fatty acid composition: 16:0 ~ 33%, 18:0 ~ 4%, 18:1 ~ 30%, 18:2 ~ 14%, 20:4 ~ 4%. The solvents were of chromatographic standard grade: chloroform, butanol and trifluoroethanol were from Aldrich (Milwaukee, WI), hexadecane was from Fluka. Potassium chloride was calcinated to remove organic impurities. Water purified by Milli-Qll (18.2 M, Millipore, USA) was used to make all solutions and in all cleaning procedures.

2.2. Methods

2.2.1. Preparation of the forming solutions

Lipid was dissolved in chloroform to prevent oxidizing and the solvent was evaporated in a stream of argon. To prepare the forming solution modified with gramicidin the peptide was added as a solution in trifluoroethanol (10 mg/1 ml) and the solvent was again removed by argon. Dried residues (phosphatidylcholine or phosphatidylcholine and gramicidin mixture) were dissolved in hexadecane–butanol mixture (10:1 by volume). The forming solutions contained PC (10 mg/1 ml of solvent system) or a GD–PC mixture (weight ratios: $1:1.0 \times 10^4$, $1:2.5 \times 10^4$, $1:5.0 \times 10^4$, $1:7.5 \times 10^4$ and $1:1.0 \times 10^5$) and were stored at 4 °C for less than a week.

2.2.2. Preparation of the bilayer membranes

Bilayer membranes were obtained as bubbles at the Teflon cap constituting a measuring vessel component. The use of hexadecane as the solvent allows obtaining membranes of thickness and capacity values similar to those of membranes formed of monolayers [11]. The thinning of the BLMs was observed visually. Capacity of membranes increased with time after bilayers formation until a steady-state value was reached some 10–20 min later. The measurements were started 20–30 min after the membranes turned completely black. The bilayers area were determined with a microscope with micrometer scale built-in in the lens and were between 4×10^{-2}–8×10^{-2} cm^2 (the values were given for the bilayers area with subtracted margin).

2.2.3. Impedance analysis

Electrochemical impedance measurements were carried out using impedance system EG&G, Princeton Applied Research, Model 388 including a potentiostat/galvanostat (Model 273), a two-phase lock-in analyzer (Model 5208), electrochemical cell and a personal computer. A 4-mV amplitude sine-wave signal perturbation was applied in the 10 mHz–10 kHz frequency range. All experiments were carried out at room temperature 20 ± 1 °C.

The self-constructed measuring vessel is depicted in Fig. 1. A syringe (1) with an external thread screw (2) and with a handwheel (3) was located in its upper part. An acid-resistant steel tube (4) with a tight Teflon piston (5) was at the other end of the screw. A connector (6) made of organic glass (polymethyl metacrylate) with a platinum current electrode (7) and a silver–silver chloride measuring electrode (8) was fixed to the syringe cone. The connector ended with a tight Teflon attachment (9). The lower part of the vessel (10) made of organic glass contained a second current electrode (11) and a second silver–silver chloride measuring electrode (12). The lateral side of the vessel was a flat glass plate allowing to observe formed membranes. The syringe, the connector, and the Teflon attachment formed a tight setup, which could be filled with electrolyte solution. The forming solution was placed at the Teflon cap tip and the setup was placed in the lower part of the measuring vessel, also filled with electrolyte solution. As a result, the Teflon attachment was immersed in the electrolyte solution, the approximate level of which (13) is marked in Fig. 1. A drop of electrolyte could be squeezed out from the cap by rotating the handwheel (3) and forming sphere (14) could be simultaneously observed under microscope. *N*-butanol contained in the forming solution dissolved in aqueous solution, and *n*-hexadecane able to wet Teflon shifted into the cap. As a result, a bilayer in the form of a bubble built of lipids was produced.

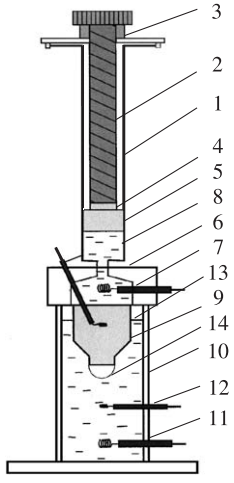

Fig. 1. The measuring vessel.

3. Theory

3.1. Impedance of gramicidin D dimers

Impedance of lipid bilayers modified with gramicidin D was measured in a system arranged in series. It is diagrammatically presented in Fig. 2 (the simplicity of the circuit reduces possible misinterpretations of the recorded data) and it is described by the equation,

$$Z = R - jX \tag{1}$$

where: Z—total impedance of the system, $j=(-1)^{1/2}$.

Effect of gramicidin on the phosphatidylcholine bilayer impedance can be determined by assuming that double layer capacity at the membrane–solution interface is unmodified by gramicidin and by distinguishing the following experimental membrane impedance components (Fig. 3).

Total impedance of the membrane is given by equation:

$$\frac{1}{R - jX} = \frac{1}{R_{PC} - jX_{PC}} + \frac{1}{R_{GG} - jX_{GG}} \tag{2}$$

A comparison of real and imaginary parts of impedances presented in Fig. 2 with those of Fig. 3 yields the relationship, which can be used for determination of impedance components, related to the presence of gramicidin channels in the membrane:

$$R_{GG} = \frac{R(R_{PC}^2 + X_{PC}^2) - R_{PC}(R^2 + X^2)}{(R_{PC} - R)^2 + (X_{PC} - X)^2} \tag{3a}$$

$$X_{GG} = \frac{X(R_{PC}^2 + X_{PC}^2) - X_{PC}(R^2 + X^2)}{(R_{PC} - R)^2 + (X_{PC} - X)^2} \tag{3b}$$

3.2. Dimerization of gramicidin D

Gramicidin channels are formed by transmembrane dimerization of the monomers originating from different monolayers composing the bilayer:

$$G + G \Leftrightarrow GG$$

Dimerization equilibrium constant of gramicidin (K_{GG}) can be written in the form:

$$K_{GG} = c_{GG}/c_G^{-2} \tag{4}$$

here: c_G—surface concentration of gramicidin monomers (mol cm^{-2}), c_{GG}—surface concentration of gramicidin dimers (mol cm^{-2}).

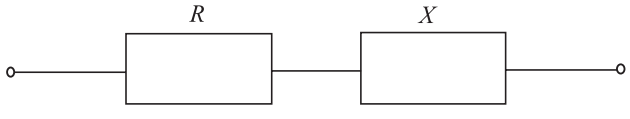

Fig. 2. Equivalent circuit representing electric properties of PC membranes modified with GD: R—real part of impedance (after subtracting electrolyte solution resistance), X—imaginary part of impedance.

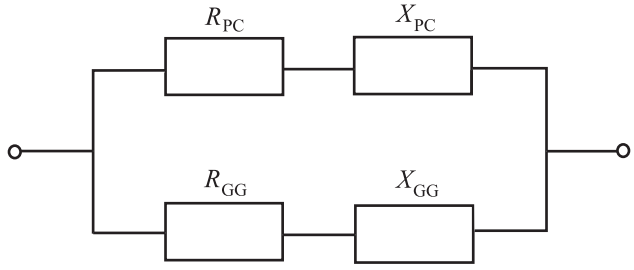

Fig. 3. Equivalent circuit of PC membranes modified with GD: R_{PC}, X_{PC}—impedance of pure phosphatidylcholine bilayer, R_{GG}, X_{GG}—impedance related to the presence of gramicidin channels able to transport monovalent ions across the membrane.

The sum of monomer (on both bilayer sides) and of dimer surface concentrations is equal to the total gramicidin concentration in the bilayer (c_T):

$$c_T = c_G + c_{GG} \tag{5a}$$

The total GD concentration can also be determined from the equation:

$$c_T = s_{PC} M_{PC}/nM_G \tag{5b}$$

in which: s_{PC}—phosphatidylcholine surface concentration in the membrane built of lipid only (mol cm^{-2}), M_{PC}—phosphatidylcholine molar weight (752.08 g mol^{-1} [12]), M_G—gramicidin molar weight (1880 g mol^{-1} [13]), n—gramicidin to phosphatidylcholine weight ratio in the forming solution.

Substitution of gramicidin monomers surface concentration obtained from the total gramicidin concentration (Eq. 5a) to Eq. (4) followed by simple transformations yield another relation to determine the equilibrium constant of GD dimerization:

$$K_{GG}^{-1/2} = c_T c_{GG}^{-1/2} - c_{GG}^{1/2} \tag{6}$$

4. Results and discussion

The experimental impedance values were related to surface area unit.

Every point of Fig. 4 represents a mean of six independent membrane measurements. Both imaginary and real parts of phosphatidylcholine bilayer impedance proved to be by two orders of magnitude lower when it was modified with gramicidin D (Fig. 4a) than those obtained from pure lipid membranes (Fig. 4b). Very simple impedance diagrams were obtained in the absence of channel-forming gramicidin D; they had the form of impedance semicircles in the entire analyzed frequency range; it was the evidence of the lipid bilayer being a dielectric layer with leakage. The semicircles were distorted because the lipid bilayer itself was not a simple and uniform dielectric layer. The dielectric was

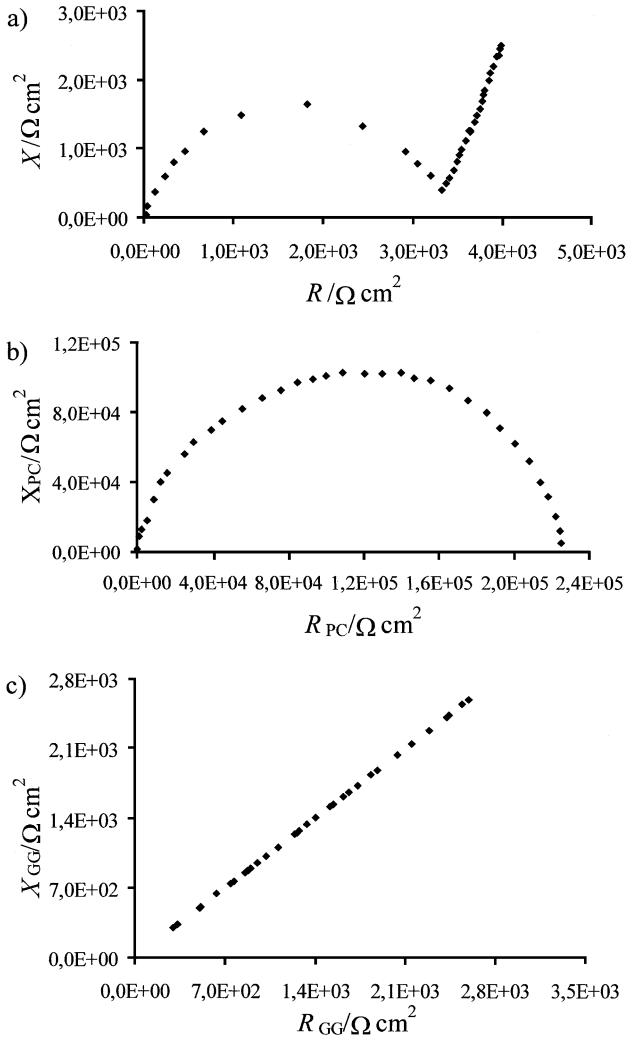

Fig. 4. Impedance spectra recorded for 1.0×10^{-3} KCl mol cm^{-3} (a) of phosphatidylcholine bilayer modified with gramicidin D (total GD concentration is equal 4.921×10^{-7} mol cm^{-2}), (b) of bilayer formed by phosphatidylcholine only, (c) of gramicidin channels ($f \geq 100$ Hz).

composed of substructures which are difficult to extract unless the phase angle can be determined separately et each frequency and very accurately. Karolis et al. [14] demonstrated the presence of seven separate elements of lipid bilayer/electrolyte systems on the ground of low-frequency impedance measurements of pure phosphatidylcholine bilayers. Four of these can be attributed to the acyl chain, carbonyl, glycerol bridge and phosphatidylcholine regions of the lecithin molecule.

The frequency response was drastically different when gramicidin was present in the membrane. The impedance spectra of the bilayer modified with GD had the form of a capacitance semicircle and, in addition, Warburg impedance appeared at frequencies lower than 2.51 Hz (for resistances greater than 3.3×10^3 Ω cm^2), probably due to potassium ion transport in the area near the membrane surface. Impedance measurements of lipid membrane were carried out in our work with unmodified membranes and with the membranes modified by five different GD concentrations and at five different KCl concentrations. Except for the Z values, all recorded impedance spectra are characterized by common general features and the same dynamic behaviour. For this reason, the data for one KCl concentration and for one GD concentration are shown in the paper (Fig. 4).

Gramicidin D introduced into a lipid bilayer readily forms dimers, which are able to transport ions if acyl chain length of membrane-forming lipids has 10–18 carbon atoms [15–17]. If the membrane thickness is not favourable for channel formation (less than 8 or more than 20 carbon atoms in the chain), then the lipid bilayer can adopt its length to gramicidin channels [18]. Very low GD amounts were added in the studies presented in this work to forming solution contained egg phosphatidylcholine; PC was mainly composed of 16- and 18-carbon fatty acid chains. The introduced gramicidin readily dimerizes in the formed bilayer allowing for the potassium ion transport. So, efficient modifying implies that fluidity of the PC membranes is sufficient to allow lateral diffusion of gramicidin monomers to form transmembrane pores by binding their terminal amino groups. Impedance of such dimers (Fig. 4c) was calculated, using Eqs. (3a) and (3b), from the impedance spectra of phosphatidylcholine membrane for the unmodified membrane (Fig. 4a) and for the membrane modified with gramicidin D (Fig. 4b).

Total gramicidin D concentration in individual forming solutions was calculated using Eq. (5b). The results are presented in Table 1 with other parameters determined as described further.

It results from Fig. 4c that the impedance due to the presence of gramicidin dimers consists of the Warburg impedance Z_W only, the impedance characteristic for the diffusion at the electrode layer which is presented by the equation [19,20]:

$$Z_W = \sigma\omega^{-1/2} - j\sigma\omega^{-1/2} \quad (7)$$

The Warburg coefficients σ modeling ion transport through the GD channels in the lipid bilayer were determined by plotting the straight lines in the $R_{GG} = f(\omega^{-1/2})$ coordinate system; the slope of the line yielded the σ value (the standard deviation was approximately 2%). The Warburg coefficient values obtained in this way in the logarithmic

Table 1
Total GD concentration in the bilayer (c_T), coverage (Θ) and surface concentration of gramicidin dimers (c_{GG}) as function of peptide/lipid weight ratio

GD/PC weight ratio	10^6 c_T/mol cm^{-2}	10^5 Θ	10^7 c_{GG}/mol cm^{-2}
$1{:}1.0 \times 10^4$	1.23	6.97 ± 0.01	9.33 ± 0.02
$1{:}2.5 \times 10^4$	0.49	2.37 ± 0.01	3.15 ± 0.02
$1{:}5.0 \times 10^4$	0.24	1.01 ± 0.01	1.36 ± 0.02
$1{:}7.5 \times 10^4$	0.16	0.55 ± 0.01	0.73 ± 0.02
$1{:}1.0 \times 10^5$	0.12	0.40 ± 0.01	0.53 ± 0.02

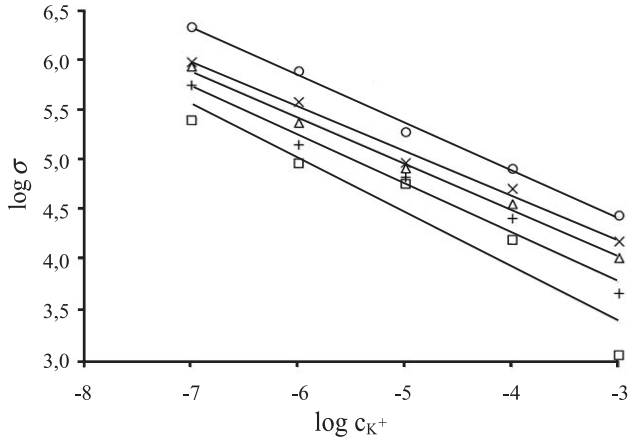

Fig. 5. Dependence of logσ on logc_{K^+}. The following total GD concentrations were used: (○) 1.230×10^{-6} mol cm^{-2}, (×) 4.921×10^{-7} mol cm^{-2}, (△) 2.406×10^{-7} mol cm^{-2}, (+) 1.640×10^{-7} mol cm^{-2}, (□) 1.230×10^{-7} mol cm^{-2}. Experimental errors are smaller than the symbols used to represent the data.

form are presented in Fig. 5 vs. potassium ion concentration logarithm (KCl concentrations in mol cm^{-3}).

The Warburg coefficient can also be calculated from the well-known equation [21]:

$$\sigma = RT/(2D)^{1/2}F^2z^2cA \tag{8}$$

here R, T, z, F are denoted as usual, D is diffusion coefficient of ion in the aqueous phase, supporting electrolyte concentration is denoted by c and membrane surface area by A.

The above equation describes the diffusion to a flat uniform surface while the ion transport across the bilayer containing GD depends on the number of open gramicidin channels. The surface fraction occupied by active GD dimers can be denoted as coverage Θ ($0<\Theta<1$). This parameter can be substituted in Eq. (8) as the membrane surface area.

Coverage values Θ were determined for PC membranes modified with various GD amounts from as the intersection points with the ordinate in the plots presented in Fig. 5 using the Warburg coefficient equation in the form:

$$\log\sigma = \log(RT/2^{1/2}D^{1/2}F^2z^2c\Theta) - \log c_{K^+} \tag{9}$$

The results are presented in Table 1.

Coverage can also be presented in the following way:

$$\Theta = c_{GG}A_{GG} \tag{10}$$

where: c_{GG}—surface concentration of the dimer (mol cm^{-2}), A_{GG}—area occupied by 1 mol of channels (cm^2 mol^{-1}).

Substituting the expression of surface concentration of the dimer obtained by transformation of Eq. (10) to Eq. (6) yields a linear dependence:

$$\Theta^{1/2} = A_{GG}c_T\Theta^{-1/2} - K_{GG}^{-1/2}A_{GG}^{1/2} \tag{11}$$

It is presented in Fig. 6.

The slope value of the straight line (Eq. (11)) is equal to the surface area occupied by 1 mol of channels. Substituting this value to Eq. (10) yields surface concentration of the gramicidin dimers (Table 1).

The area occupied by single gramicidin dimer A_G can be readily calculated if the surface area occupied by 1 mol channels is known. The A_G value obtained in this way is 124 ± 1 Å^2 molecule^{-1}. No unequivocal determination of the area occupied by gramicidin molecule has been presented in literature. The proposed values range from 120 to 150 Å^2 molecule^{-1} [22–26], and even to 250 Å^2 molecule^{-1} [27]. Divergences in the surface area values can be due to the formation of closely packed clusters, the transition from a (single-stranded) monomeric molecule conformation to a (double-stranded) dimeric component or other conformational changes, the reorientation of parallel molecules to perpendicular with respect to the interface, the formation of multilayered structures as suggested by Malcolm [28] for hydrophobic synthetic peptides. Molecular areas deduced from space-filling models of double-stranded (ds) helices such as ds β^6 and ds β^7 are 100 and 150 Å^2 in perpendicular orientation [29]. Analogously, the areas deduced from the models of single-stranded (ss) helices like ss $\beta^{4.4}$ and ss $\beta^{6.3}$ are 150 and 215 Å^2 molecule^{-1}, respectively [30,31]. However, the molecule surfaces can overlap in the case of close packing resulting in drastic area reduction. 162 Å^2 molecule^{-1} was found for gramicidin in a closely packed pentameric array of ss $\beta^{6.3}$ [32]. Potential reason of the variation occurring in the π–A curves

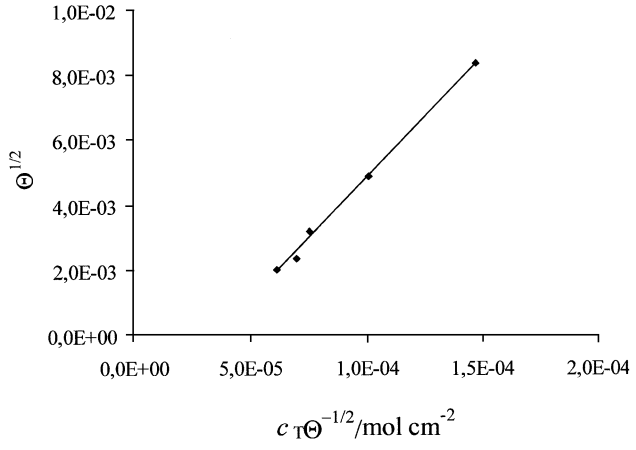

Fig. 6. The plot illustrating Eq. (11) used to determine A_{GG} and K_{GG} parameters. Experimental errors are smaller than the symbols used to represent the data.

in molecule surface area determination is the evaporation of solvent and the adherence of the peptide to the syringe tip used to apply the material to the interface. This can result in marked variations in the π–A curves and in an underestimation of the molecule area. Reproducibility of the π–A curves can be improved by coating of the syringe tip [32]. Additional reasons of the errors of compression isotherm analyses are different collapse pressure definitions.

The area occupied by gramicidin monomers dispersed in a PC bilayer remains the same during dimerization process. Thus, the area occupied by a gramicidin dimer calculated by us can be compared with the range of values proposed by other authors regarding the surface of gramicidin monomer.

Intersection of the straight line with the ordinate (Eq. (11)) yields the equilibrium constant of gramicidin dimerization equal to $(1.06 \pm 0.12) \times 10^7$ cm^2 mol^{-1}. This K_{GG} value is similar to the values proposed by other authors, carrying out from 10^8 to 10^{17} cm^2 mol^{-1} [33,34].

5. Conclusions

Separation was proposed of the ion transport effect by gramicidin channels in the equivalent circuit by presenting it as the separate branch. This separation was proved to be correct by analysis of the experiments. The branch corresponding to the effect of gramicidin channels in the equivalent circuit contains Warburg impedance only, probably due to transport of potassium ion in the area near to the membrane surface. Determined gramicidin channel parameters such as area occupied by a single dimer and equilibrium constant of gramicidin dimerization are in agreement with the values given by other authors.

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