The Study of Volt-Ampere Characteristics of Ionic Channels Formed by Gramicidin A

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Abstract. A method of measurement of the non-linearity coefficient of volt-ampere characteristics of the type i $(U) \sim U(1+\beta U^2)$ has been developed for ionic channels formed by gramicidin A, using the third harmonic of the membrane current. The shape of the volt-ampere characteristics (VA) of ionic channels formed by gramicidin A did not depend on the antibiotic concentration in the membrane. The coefficient β of non-linearity of VA of membranes modified by gramicidin A depended on electrolyte concentration "c" and it increased proportionally with the lg c from $-17\ V^{-2}$ at 0.03 mol/l KCl to $8\ V^{-2}$ at 3.4 mol/l KCl, and it was zero at $c_0 = 0.3 - 1$ mol/l KCl. Egg lecithin and glycerolmonooleate (GMO) membranes differ in their c_0 values. The substitution of K^+ for Li^+ of the membrane solvent (n-heptane for n-hexadecane) did not influence the value of β; the same applied for GMO membranes without any solvent. In a number of membranes, spontaneous change of the non-linearity coefficient with time observed after the membrane formation, as well as jumps of the non-linearity coefficient at a practically unchanged membrane conductivity. An analysis of some theoretical models of the ion transport through the channel has shown that, at voltages above 200 mV, these models provide rather small values of β, or extremely high VA non-linearity.

Key words: Ion channels — Gramicidin A — Bilayer lipid membranes — Volt-ampere characteristics — Non-linearity coefficient

Introduction

Results of the determinations of the properties of model ion channels (ICH) can be used to compare them with experiment of theoretical models of ion transfer through ionic channels in biological membranes. Ionic channels formed by gramicidin A (GRA) (Haydon and Hladky 1972; Hladky and Haydon 1972) are most

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common used. Conductance of bilayer lipid membranes (BLM) with incorporated ionic channels, current fluctuations, ion selectivity, etc. may all be measured. A number of other parameters, such as the channel conductance, can conveniently be determined in a simple ionic channel; however, such methods of measurements are not suitable for accurate determination of the volt-ampere characteristics (VA) as the ionic channels are in the conductive state short periods of time (fractions of seconds) only. At the same time, however, the measurement of the shape of VA of membranes with a high number of ionic channels is associated with difficulties due to the number of simultaneously open channels changing during measurement. As a result the VA shape may become distorted. Therefore, for a detailed study of the VA shape and comparison of the results obtained with theoretical models it is first of all necessary to develop a special method which would allow to determine the VA shape of a simple ionic channel on the basis of the measurement of properties of modified membranes.

Early studies of ionic channels formed by GRA have already established that the VA of GRA-modified membranes are non-linear. The non-linearity depends on electrolyte concentration and composition (Hladky and Haydon 1972). However, so far, no quantitative characteristics of the VA non-linearity have been available, probably due to the fact that the hitherto used methods of VA recording in simple channels have rather be inconvenient, and that the deviation of the GRA channels VA from straight line is small and its determination by a graphic method is rather difficult. We have developed a method (Flerov et al. 1981) which allows direct measurement of non-linearity requiring no preliminary registration of the VA shape. The method consists in the measurement of higher harmonics of currents generated in membranes modified by GRA (Passechnik and Hianik 1978) or dipicrylamine (Carius 1979). It has been found that the registration of VA non-linearity using this method enables investigation of mutual interactions of ionic channels of changes of the kinetics of membrane properties during the formation of the membrane, and that it offers a possibility to verify the theoretical models of ion transfer through the GRA channels.

Methods

1. Non-linearity measurements using the third harmonic

The current i flowing through a modified membrane depends on both the voltage supplied to the membrane — U, and on changes of this voltage with the time volt-ampere relationship with a slight hysteresis can be obtained in recording membrane VA. Similar phenomena will be discussed later; now we shall assume that the current is determined by voltage alone, i. e. VA of the membrane $i = i(U)$. We shall investigate symmetric VA, i. e. BLM for which $i(U) = -i(-U)$. In the first approximation according to the U powers such VA can be expressed as follows:

$$i(U) = gU(1 + \beta U^2), \tag{1}$$

where g stands for the conductivity of the membrane, and β — is a variable termed VA non-linearity coefficient. Let a voltage of a frequency $\omega/2\pi$: $U = U_0 \sin \omega t$ be supplied to the membrane, then the current through the membrane will be

$$i(t) = gU_0 \sin \omega t + g\beta U_0^3 \sin^3 \omega t + \frac{\mathrm{d}}{\mathrm{d}t}(CU_0 \sin \omega t). \tag{2}$$

Let us first assume that the membrane capacity C does not depend on the voltage U i. e. the membrane is incompressible. Then, the following relationship can be derived from Eq. (2):

$$i(t) = \left(gU_0 + \frac{3}{4}g\beta U_0^3\right) \sin \omega t + CU_0\omega \cos \omega t - \frac{1}{4}g\beta U_0^3 \sin 3\omega t. \tag{3}$$

Let us now consider the capacity change in dependence on U. In a previous study (Passechnik and Hianik 1977) it was shown that the membrane compression by the electric field results in the generation of complementary components in the membrane current:

$$i_E = \frac{3}{4}\frac{C_s U_0^3}{hE_\perp}\omega C(\cos \omega t - \cos 3\omega t), \tag{3'}$$

where $E_\perp$ is the Young modulus of elasticity of the membrane in the direction perpendicular to its surface; C_s is the specific capacity; and h is membrane thickness. Thus, the current through the membrane will comprise the basic and the third harmonics of the frequency ω:

$$i = i_1 + i_3 \tag{4}$$

where

$$i_1 = \left(gU_0 + \frac{3}{4}g\beta U_0^3\right) \sin \omega t + CU_0\omega\left(1 + \frac{3}{4}\frac{C_s U_0^2}{hE}\right) \cos \omega t \tag{5}$$

$$i_3 = -\frac{1}{4}g\beta U_0^3 \sin 3\omega t - \frac{3}{4}\frac{C_s U_0^3}{hE}\omega C \cos 3\omega t \tag{6}$$

To neglect the capacity components in the above relationship following conditions must be met:

$$g\beta \gg \frac{3C_s\omega C}{hE_\perp}, \qquad g \gg \omega C \tag{7}$$

We shall evaluate the effect of the capacity components. In our experiments an alternate voltage $U_0 \approx 150$ mV with a frequency of about 40 Hz was used. The Young membrane elasticity modulus used in our experiments was not smaller than 10^7 Pa (Hianik 1979). Thus, the variable $\frac{3}{4}\frac{C_s U_0^2}{hE_\perp} \approx 10^{-3}$. As will be shown later, under our conditions $|\beta| U_0^2 = (1 \div 20) \cdot 10^{-2}$. Thus, both conditions (7) will be satisfied if $g \gg \omega C$, i. e. if the specific conductance of the membrane $g_s > 1$ Sm/m^2 at $\omega/2\pi = 40$ Hz. These are experimental conditions that may easily be meet and the effect of the capacity currents on the current first harmonic value can be neglected. The influence of capacity components on the third harmonic current can already be neglected at approximately $g_s > 0.1$ Sm/m^2 (depending on the variable β). Thus, we get

$$\begin{aligned} i_1 &\approx gU_0 \sin \omega t = A_1 \sin \omega t \\ i_3 &\approx -\frac{1}{4}g\beta U_0^3 \sin 3\omega t = A_3 \sin 3\omega t \end{aligned} \tag{8}$$

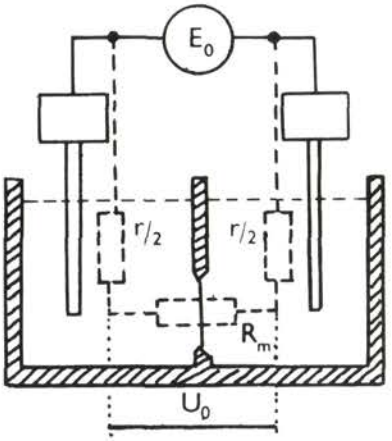

Fig. 1. Influence of the electrolyte resistance on β measurement. The generated voltage E_0 is divided between the resistance of the circuit r and the resistance of the membrane R_m. U_0 — membrane voltage.

Into the expression (8) we have introduced the amplitudes of both the current first and third harmonics A_1 and A_3. The first harmonic current is in coincident phase with voltage U; at the same time, however, the phase shift between the third harmonic current and the voltage can be 0° or 180°, depending on the β sign. A corresponding phase shift will be recorded in terms of the sign of the amplitude of the third harmonic A_3. Cases in which the phase shift differes from these values will be discussed later. The non-linearity coefficient is expressed through the amplitude of the membrane current first and third harmonics

$$\beta = -\frac{4}{U_0^2}\frac{A_3}{A_1} \tag{9}$$

If the non-linearity coefficient of the membrane VA β does not change during the experiment and if the U_0 value is constant then the amplitudes of A_3 and A_1 are mutually proportional. Thus, if we measure the amplitude of the voltage U_0 supplied to the membrane, and the amplitudes of the harmonics of membrane currents A_3 and A_1 with their phases, we can determine the non-linearity coefficient of the membrane VA, β.

2. Effect of electrolyte resistance

Relationships (2) — (9) are applicable to case when the value of the voltage U applied directly to the membrane is known. However, in an actual experiment, the sinusoid voltage from the generator with amplitude E_0 is supplied to the membrane through the measuring system, electrodes and the electrolyte surrounding the membranes — Fig. 1 (the resistance of the measuring system is given by the output resistance of the generator and the input resistance of the measuring amplifier). Voltage E_0 is partly reduced due to an additional resistance r (system-electrodes-electrolyte), represented primarily by the electrolyte resistance (to 10—20 kΩ). Only voltage E_0 can be measured, the voltage of the membrane itself, U_0 is not accessible for direct measurement. We shall now study changes of the relationship (2) — (9) if the measured parameter E_0 is considered instead of U_0.

The generator voltage E_0 becomes divided between the resistance of the circuit r and the resistance of the membrane R_m:

$$E_0 = i \cdot r + U_0 \tag{10}$$

We eliminate U_0 from the system of equations (1) — (10) and we get the dependence $i(E_0)$. $U_0 = E_0 - ir$, and thus:

$$i = g(E_0 - ir)\,[1 + \beta(E_0 - ir)^2] \tag{11}$$

We shall assume that β is a small value (i. e. $\beta E_0^2 \ll 1$) and we shall solve the equation (11). Let us develop the current i into a series according to βE_0^2, i. e. let $i = i^{(0)} + \beta E_0^2 i^{(1)} + \ldots$. Then, from Eq. (11) with precision to $\beta^2 E_0^4$ we get:

$$i^{(0)} + \beta E_0^2 i^{(1)} = g(E_0 - r i^{(0)} - r\beta E_0^2 i^{(1)}) \cdot [1 + \beta(E_0 - r i^{(0)} - \beta E_0^2 r i^{(1)})^2] \tag{12}$$

When comparing the coefficient at the same powers in the equation (12), we get

$$\begin{aligned} i^{(0)} &= g(E_0 - i^{(0)} r) \\ E_0^2 i^{(1)}(1 + rg) &= g(E_0 - r i^{(0)})^3 \end{aligned} \tag{13}$$

$$i^{(0)} = \frac{gE_0}{1 + rg}, \quad i^{(1)} = \frac{gE_0}{(1 + rg)^4} \tag{14}$$

and finally

$$i(E_0) = i^{(0)} + \beta E_0^2 i^{(1)} = \frac{gE_0}{1 + rg}\left[1 + \frac{\beta E_0^2}{(1 + rg)^3}\right] \tag{15}$$

By comparing equations (1), (9) and (15) we establish that the coefficient of VA non-linearity and the membrane conductance are now determined from relationships

$$g = \frac{A_1}{E_0 - rA_1}, \quad \beta = -\frac{4}{E_0^2}\frac{A_3}{A_1}(1 + rg)^3 \tag{16}$$

We can see that the correction $(1 + rg)$ enters the relationship for β as the third power. This means that in spite of the low value of the resistance r as compared with the resistance of the membrane $1/g$, the effect of the former on the amplitude of the third harmonic can be significant. The resistance r is difficult to be accurately measured, and at high conductances of the membrane ($g \sim 1/r$) the accuracy of the determination of β is remarkably reduced. Practically, it is more convenient to calculate the measured quantity A_3 to the quantity A_3^{kor}, which is determined from the relationship $A_3^{kor} = A_3(1 + rg)^3$. In this case, we get a more convenient relationship for β which does not comprise the electrolyte resistance:

$$\beta = -\frac{4}{E_0^2}\frac{A_3^{kor}}{A_1} \tag{17}$$

3. The effect of voltage change rate on BLM

As already shown under methods 1., the current through the membrane depends on the nature of voltage changes with the time. This is due to the fact that the membrane conductance $g = \lambda N_2$ is determined by both the number of open channels N_2 and the conductance of the channel λ. Also it is

known that for GRA, the probability of the channel open state obviously depends on the voltage supplied (Haydon and Hladky 1972). As changes in the number of channels is in delay to voltage changes on the membrane, the current is in delay to the voltage, and when recording the membrane VA hysteresis curve is obtained. This means that the non-linearity of the membrane VA is thus determined by the non-linearity of the channel VA as well as by the change in the number of channels with the change of the voltage. Measurement of VA non-linearity is made easier also due to the fact that the change in the number of channels is a slow process. For GRA, the time constant of this process is about 0.5 s (Bamberg and Läuger 1973). Therefore, when supplying alternate voltage to the membrane hysteresis will be observable only at low frequencies (0—5 Hz). In accordance with this, at the above frequencies the VA shape will depend on the voltage frequency. Therefore, we have first studied the dependence of the membrane VA on frequency in a separate experiment, prior to the measurement of the harmonics. Harmonics were then measured at frequencies at which the VA shape did not depend on the frequency, i. e. within an interval of frequencies not below 20—40 Hz. At these frequencies, the number of open channels, N_2, cannot be substitued for by the period of alternate current and is determined by the mean value of the voltage for the period. Consequently, the coefficient of non-linearity β, measured at these frequencies, is determined only by the non-linearity of the channel conductance and not by the number of the channel in the membrane. Thus of different methods which allow controling the number of channels in the membrane (see Eisenman et al. 1980) we used a method which is based on the application on the membrane of alternating current with a frequency of 40 Hz.

4. On pecularities of the parameter β

The parameter β is in fact determined by the same characteristics of the channel as its conductance — the potential profile and ionic composition of the electrolyte. However, it is more suitable to interpret the results obtained during the study of the channel according to the parameter β than according to other parameters of the channel. Namely, the membrane conductance as well as some other parameters e. g. unidirectional ionic currents, current fluctuations are additive parameters. Thus, if one of these parameters is recorded, the question arises whether it was the channel parameter itself, or the number of channels that changed. This leads to enunivocal interpretation of some results. The coefficient of non-linearity of the membrane VA, however, is not an additive parameter: if there are channels with different coefficients of non-linearity in the membrane, then the non-linearity coeficient of the whole membrane shall not be the sum but the mean value of the non-linearity coefficients of individual channels. Therefore, if a change of β is observed only one conclusion is possible: in some or all channels the non-linearity coefficient has changed. No other factor, such as change in the number of the channels, change of the membrane area, etc. can influence β.

Experimental

1. Measurement of the non-linearity coefficient β

A block diagram of the apparatus is shown in Fig. 2. The alternate voltage E of a frequency of $\omega/2\pi$ measured using a frequency meter (2) was supplied from the generator (1) with a low coefficient of harmonic distortion through electrodes (3) and (4) to the membrane (5). The total current through the membrane was amplified by the amplifier (6), rectified by the detector (8) and fed to the input X of the XY plotter — (10). The third harmonic of the current was amplified by a resonance amplifier (7) tuned to the frequency of 3ω, and after rectification with

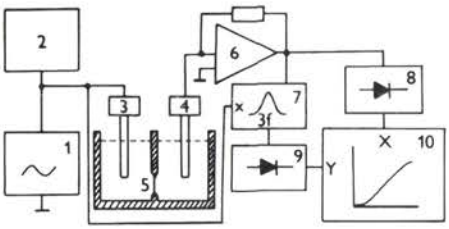

Fig. 2. A block diagram of the apparatus for the measurement of the non-linearity coefficient β. For explanation, see the text.

a detector (9) it was fed to the input Y of the plotter. As a remarkable portion of the current flowing through the membrane consists of the current of the first harmonic, the plotter draws a curve in coordinates A_3 (A_1). The built-in oscillographic indicator of the resonance amplifier (7) was synchronized by voltage from the generator (1). Based on Lissage pictures the phase A_3 could be determined with respect to E. The noise of the apparatus corresponded to the value of $|\beta| < 0.5\ V^{-2}$; using other resonance amplifiers it is however possible to improve the parameters of the apparatus quite easily (no measurement of lower β values was required in our experiments).

The measurement of the resistance r required for correct quantification of values of β, was carried out by the following method. The value of r includes the resistance of electrodes (0.5—2 kΩ) and that of electrolyte in the measuring cell (1—20 kΩ). The electrolyte resistance does practically not depend on the geometry of the siting of the electrodes in the cell and is determinded only by the electrolyte layer in the immediate vicinity of the membrane (~ 1 mm). This means that the given resistance practically depends only on the membrane surface. As the membrane surface in our experiments was in fact equal to the surface of the cell opening (the border area was smaller than 5 % of the surface), we though it possible to measure the resistance r with an ohmmeter through electrodes on the cell without membrane. Measurements of r directly on the cell with a membrane (e. g. when applying voltage of a high frequency to set $1/2\pi fC \ll r$), were found to be less accurate.

2. Recording of the volt-ampere relationship of membranes

In addition to the measurement of the VA non-linearity coefficient we have also recorded VA itself using a memory-oscilloscope. Measurements were carried out with sinusoidal voltage or saw tooth voltage applied to the membrane. In the first case (Fig. 3*A*) the sinusoidal voltage from generator (1) was fed to the membrane and to the input X of the memory oscilloscope (3), in X—Y mode corresponding to

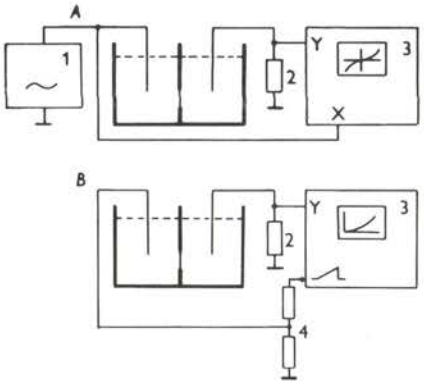

Fig. 3. A block diagram of the experimental lay-out for the registration of the membrane VA. *A*: sinusoidal voltage prolocoll *B*: saw-tooth impulse prolocoll. For symbols, see text.

the operation mode of the XY plotter. The current through the membrane passed through a measuring resistor (2), the voltage from which was recorded at the input Y of the oscilloscope.

In the second case the oscilloscope (3) was working in normal mode of linear synchronization — Fig. *3B*. The voltage from the synchronization generator was applied through a divider (4) to the membrane. The current through the membrane was measured in the same way as in the first case. This method was found very efficient when using single synchronization. In this case, the membrane was under zero voltage for a longer period of time till the impulse application. When using sufficiently short independent saw tooth pulses (~ 10 ms) it was possible to obtain VA of membranes the voltage range from 0 to 300 mV without electrical break down of the membrane.

3. Experimental procedures

Membranes were formed in electrolyte solution with previously added GRA. The formation of the membrane was checked using the first and the third harmonic of the current (see, e. g. section I, Fig. 5*A*). The phase of the third harmonic with respect to E was approx. — 90°. During the incorporation of the modifier into the membrane, A_3 and A_1 increase and the phase i_3 is 0° or 180° (depending on the sign of β) — see section II, Fig. 5*A*. During the experiment the electrodes were sometimes disconnected from the scheme (Fig. 2) and for the VA study and photographing they were temporarily connected to the scheme shown in Fig. 3*B*.

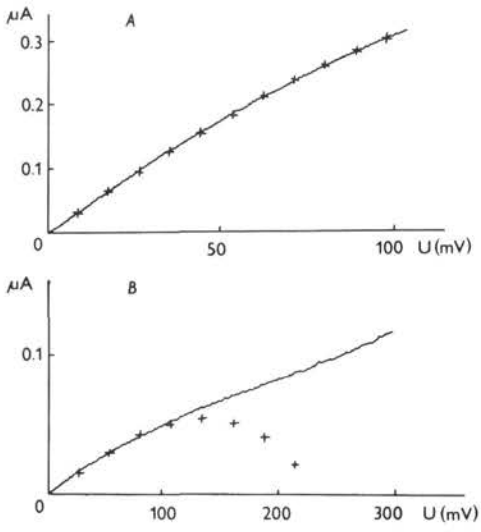

Fig. 4. Volt-ampere characteristics of GRA-modified membranes. Application of independent saw-tooth pulses (10 ms). Membrane: glycerolmonooleate in n-hexadecane, electrolyte: 0.1 mol/l LiCl. Crosses: approximation in the points 0; 50 and 100 mV by Eq. (1). *A*: voltage amplitude — 100 mV, approximation parameters: $g = 3.6 \times 10^{-6}$ Sm, $\beta = -15.5$ V^{-2}. *B*: voltage amplitude −300 mV. $g = 5.4 \times 10^{-7}$ Sm, $\beta = -14.3$ V^{-2}.

Verification of the measurements of β using the third harmonic

In deriving relationships (1) — (16) several assumptions were required. Their validity for the given experimental conditions should be verified:
a. to which degree the membrane VA are determined by relationship (1);
b. at which membrane conductances the capacity components in relationship (4) can be neglected; *c*. what are the conditions under which the effect of the electrolyte resistance is sufficiently low and which factors may limit the precision of the method used; *d*. at which frequencies the number of channels does not depend on the voltage applied.

In other words, it is necessary to evaluate the exactness of the method and to compare it with other methods studying membrane VA.

1. The shape of volt-ampere characteristics of GRA ICH

The same membrane was used to measure the first and the third harmonic of the membrane current as well as the volt-ampere characteristics upon the application of a 10 ms saw tooth pulse (Fig. 4*A*). The time interval between these two

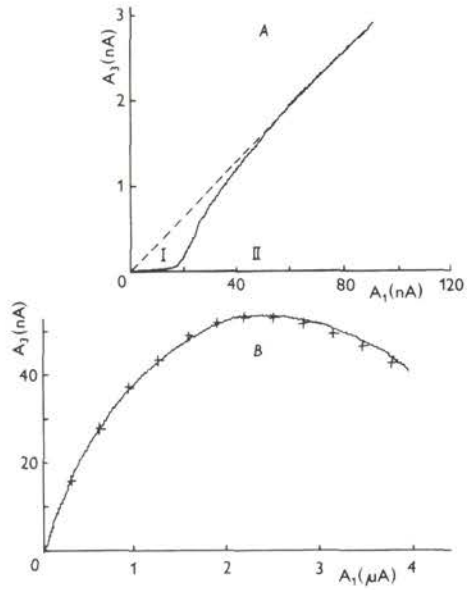

Fig. 5. The dependence $A_3(A_1)$ for low (A) and high (B) membrane conductances. *A*: glycerolmonooleate in n-hexadecane; 0.1 mol/l LiCl. It is possible to determine the effect of the capacitance on the harmonic of the current. *B*: glycerolmonooleate in n-hexadecane; 0.1 mol/l LiCl. Crosses represent an approximation of calculated according (16) with parameters: $\beta = -21.5\ V^{-2}$, $r = 10.7\ k\Omega$ (see text).

measurements did not exceed 30 s. The obtained VA was then approximated by a polynomial (1) for points 0; 50; 100 mV. The result of the approximation is shown in Fig. 4*A* (crosses). It was found that membrane VA are well approximated up to the voltage of 100 mV by the relationship (1) with a parameter $\beta = -15.5 \pm 4.0\ V^{-2}$. Based on the results of the measurement of harmonics and using relationship (16) we have obtained $\beta = -11.5 \pm 0.7\ V^{-2}$. Thus, similar values of the parameter are obtained by different methods; however, the determination of β on the basis of the third harmonic of the current is much more accurate and quicker than that derived directly from the membrane VA.

Measurements within a larger voltage interval have shown that at voltages $E > 100$ mV membrane VA are no more approximated by relationship (1). Fig. 4*B* gives VA up to a voltage of 300 mV and its approximations in points 0; 50; and 100 mV. In such cases, a more comprehensive relationship should be used instead (1):

$$i(U) = gU(1 + \beta U^2 + \beta_2 U^4 + \ldots) \tag{18}$$

It may be shown (analogically to relationship (3)) that the incorporation of the member $\beta_2 U^4$ indicates the occurrence of the fifth harmonic in the membrane

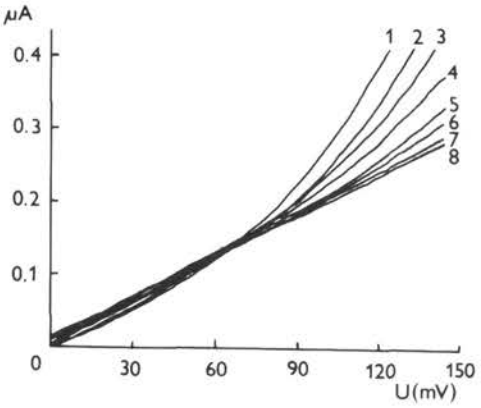

Fig. 6. VA characteristics of GRA-modified membranes at different rates of voltage changes when applying saw-tooth voltage of different duration: 1 — 1.5; 2 — 15; 3 — 30; 4 — 75; 5 — 150; 6 — 300; 7 — 750; 8 — 1500 mV/s.

current. This harmonic was recorded at a voltage $E_0 = 140$ mV on the egg lecithin membranes in n-heptane (frequency 40 Hz, electrolyte 0.02 mol/l KCl). This phenomenon was previously observed only under different conditions (frequency 376 Hz, electrolyte 0.1 mol/l KCl) (Hianik et al. 1979). The amplitude of the fifth harmonic was by one order of magnitude smaller than that of the third harmonic. The value of the fifth harmonic can be used to check the accuracy of relationship (1) in expressing the membrane VA and thus, whether relationships (1) — (16) are applicable.

2. The effect of membrane capacitance and electrolyte resistance

It is evident from relationships (5), (6), (16) that, at high BLM conductances g, it is possible to ignore membrane capacitance C, and at small conductances — the resistance of electrolyte r. However, due to the gradual incorporation of the antibiotic into the membrane, the conductance of the later gradually increases. Therefore, both the capacitance C and the resistance r at different stages will affect the shape of the curve $A_3(A_1)$.

Fig. 5*A* illustrates the initial part of the relationship $A_3(A_1)(E_0 = 100$ mV, frequency 40 Hz). In segment I, membrane formation starts and no GRA channels have yet been incorporated (the value of A_1 increases with that of A_3 changing insignificant). The third harmonic is determined by membrane electrostriction and is shifted to $\sim 90°$ with respect to voltage E. The first harmonic is determined by the membrane capacitance C. After the incorporation of GRA, A_1 and A_3 are increasing — segment II, phase A_3 equals 0° or 180°. This means that the graphical

dependence of A_3 on A_1 can be used to determine the effect of the capacitance on the harmonic of the current and to consider it in computing β according to the ratio A_3/A_1.

Fig. 5*B* shows the dependence of $A_3(A_1)$ at a membrane conductance g, comparable with $1/r$; crosses represent a curve approximating the relationship derived from relationship (16) with parameters $\beta = 21.5\ V^{-2}$, $r = 10.7\ k\Omega$. It is evident that the drop of the A_3 value at growing membrane conductance is in good agreement with the theoretical depencence (16). The position of the aximum enables to determine r, or — as can be shown — the maximum corresponds to the membrane conductance $4/r$.

This means that, within a rather large interval of BLM conductances both the membrane capacity and electrolyte resistance can be neglected (e. g., within the interval of 3×10^{-7} Sm to 3×10^{-5} Sm for typical values of $\beta = \frac{-}{+} 10\ V^{-2}$, $r = 10\ k\Omega$, frequency 40 Hz).

3. The effect of frequency ω

VA characteristics of GRA modified membranes were measured at different rates of voltage changes (when applying saw-tooth voltage of different duration) (Fig. 6). It is evident from Fig. 6 that at rates of 1.5 mV/s a strong non-linearity of the membrane VA may be observed caused by the dependence of the number of open

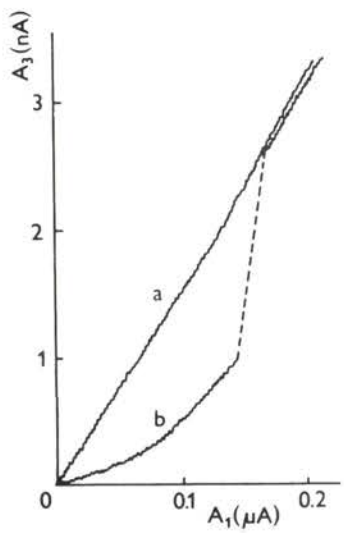

Fig. 7. The dependence $A_3(A_1)$. Glycerolmonooleate in n-heptane; 0.3 mol/l KCl, $E_0 = 100$ mV. *a* — normal behaviour of the membranes; *b* — anomalous behaviour of the membranes.

channels on the voltage on the membrane (Bamberg and Läuger 1974) — (curve 1). With the increasing rate of voltage changes, the non-linearity is reduced (curves 2,3,4) and at rates higher than 750 mV/s, the shape of the curve remains unchanged. Thus if voltage changes occur with frequencies higher than 200 ms, the opening of ionic channels does practically not affect membrane VA. It is then determined practically only by the VA shape of the ionic channel. Therefore, to measure VA non-linearity coefficient higher frequencies higher than 5—10 Hz should be used ($2\pi/\omega \ll 200$ ms). No marked increase in the frequency is desirable as it would result in a growth of the capacity current (4). A frequency reduction below 20 Hz is not desirable either as it results in an increase of the noise of the device. Individual experiments have shown that volt-ampere characteristics measured using sinusoidal voltage are practically the same for frequencies from 20 to 10^3 Hz. Because of that most of our experiments were carried out at a frequency of 40 Hz.

VA non-linearity of gramicidin A channels

1. Dependence of the VA non-linearity on the concentration of ionic channels

Usually it is assumed that GRA channels operate in the membrane indepedently and that an increase in the membrane conductance is associated with an increased number of channels in the membrane. However, some experimental results seem to argue against the above statement. In a study by Kolb and Bamberg (1977) a significant non-linearity of the dependence of the membrane logarithm electric noise on the logarithm of its conductance was observed at high membrane GRA concentrations (membrane conductance was about 100 Sm/m^2 in 1 mol/l CsCl). This has led the authors to an assumption concerning interaction of the channels for high membrane GRA concentrations. Assuming that interactions of channels should affect the non-linearity coefficient of the ionic channels we carried out special measurements of β at high membrane conductances.

We have measured β within a large interval of membrane conductances. We have used membranes from glycerolmonooleate (Sigma) in 20 mg/ml n-hexadecane (of chemical purity grade), electrolyte 0.1 mol/l KCl, $E_0 = 100$ mV, frequency 40 Hz. For each of the membranes values of A_3 and A_1 were recorded since the beginning of its formation till its bursting. During this time the membrane conductance was continously increasing due to incorporation of GRA from the electrolyte. It was found that the dependence of A_3^{kor} on A_1 was practically linear (curve *a*, Fig. 7), i. e. $\beta = \text{const}$. In order to further extend the studied interval of the membrane conductances we have carried out a series of experiments in which different concentrations of GRA were used. We have calculated the value of A_3^{kor}

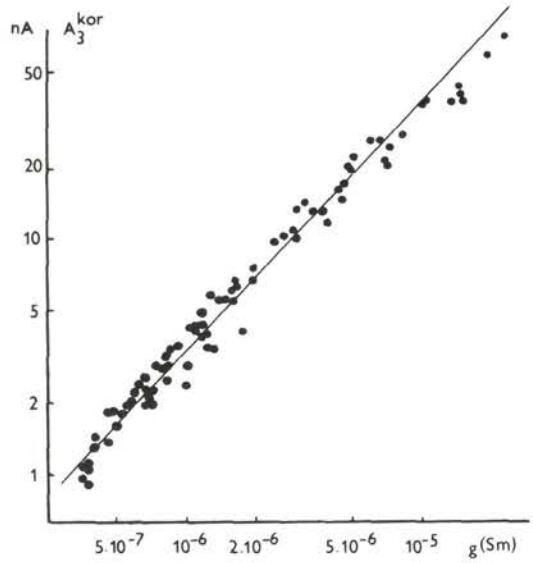

Fig. 8. The dependence $A_3^{kor}(g)$ of GRA-modified membranes. Glycerolmonooleate in n-hexadecane; *0.1 mol/l* LiCl, $E_0 = 100$ mV. *Correlation coefficient*: 1.015 ± 0.020.

and g at two points — at the beginning and at the end of the process of GRA incorporation for every membrane. These points were plotted as shown in Fig. 8. It can be seen that dependence of A_3^{kor} on g was practically linear up on a change of the membrane specific conductance from 0.23 to 56 Sm/m^2. This means that $\beta = \text{const.}$ over the entire interval of conductances.

An entirely linear dependence of A_3^{kor} on g was also observed for glycerolmonooleate membranes in n-heptane and egg lecithin membranes in n-heptane (20 mg/ml) in KCl at concentrations from 0.01 mol/l to a saturated solution and LiCl from 0.03 mol/l to 1 mol/l.

This means that no deviations from the principle concerning the independence of the effect of GRA channels could be observed in spite of the fact that the interval of GRA channel membrane concentrations used included concentrations used in the study by Kolb and Bamberg (1977). It might be assumed that the latter study did not consider the resistance of the electrolyte. We have found that a correction of the results reported in the above paper, for electrolyte resistance (1—4 kΩ) analogically to relationships (10) — (16) allows to set a completely straight line for the noise conductance dependence, i. e. to ascribe it to purely additive inputs from single ionic channels.

It should be noted that with nine membranes (6 % of the total number of glycerolmonooleate membranes in n-hexadecane or n-heptane) we have observed "anomalous" behaviour of the membrane — (curve *b*, Fig. 7). For these membra-

nes, the value of β at the beginning of the incorporation of the channel was significantly lower than the "normal" value; in the process of incorporation of channels into the membrane, A_1 and A_3 increase, the dependence of A_3 on A_1 is, however, non-linear. A few minutes after the formation of the membrane, A_3 suddenly increases to a "normal" value — (curve *b*, Fig. 7), although the conductance g is only little changed. Let us now discuss which might underlay these changes.

It is known that the membrane properties continue to change for a longer time interval after its blackening (Sargent 1975). At the very beginning of the BLM formation the dispersion of its properties can be quite remarkable. One can assume that in some cases during the formation of the membrane, membranes with different properties are formed first (e. g., with a high number of micro-lenses). The properties of the membrane are changing very slowly first, and then the membrane becomes "normal" through a jump. If, in such changed membranes, channels have different VA, we get a change of non-linearity of the whole membrane over time, analogically to the observed phenomenon (curve *b*, Fig. 7). This can also explain why such membranes are observed only rarely — 6 % of the total number of membranes. We should emphasize that the change in BLM conductance is small at such jumps, and can hardly be recorded when measuring only membrane conductance alone. The phenomenon of the change of the value for GRA channels undoubtedy calls for further study.

2. *Depencence of VA non-linearity on BLM and electrolyte composition*

We have measured the β non-linearity coefficient of GRA-modified membrane VA with different lipids (glycerolmonooleate and egg lecithin), membrane solvent (n-heptane, n-hexadecane), composition of electrolyte (KCl and LiCl). In all cases, we have measured the dependence of β on the electrolyte concentration (from 0.01 to 3 mol/l). The results are shown in Fig. 9. Each point represents the mean value and mean quadratic error calculated from the series of 4—6 membranes formed subsequently. Differences in β values between different series may reach approximately 150 %, therefore the actual values of the errors may be somewhat higher than those given in Fig. 9.

It is evident that for all membranes of different structures the β value increases with the increasing concentration of electrolyte c, and β is proportional to lg c. For small c the VA shape is sublinear (i. e. the current increases more slowly than voltage, $\beta<0$) at $c_0\approx 0.3-1$ mol/l is linear ($\beta=0$) and for $c>1$ mol/l it is superlinear (current increases more quickly than voltage, $\beta>0$) (see inset in Fig. 9).

The channel conductance and life time are strongly influenced by factors such

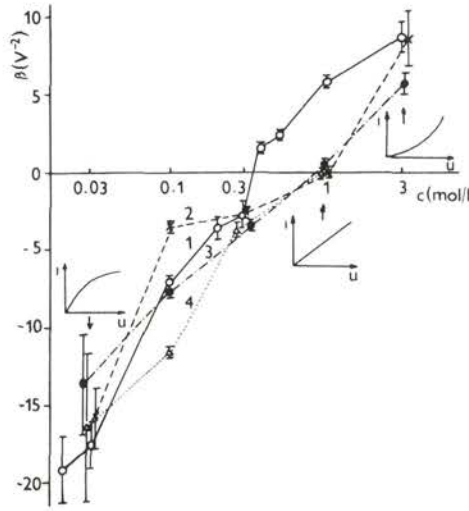

Fig. 9. The dependence of VA non-linearity on the BLM composition and electrolyte concentration c. $E_0 = 100$ mV, frequency 40 Hz. The shapes of the VA characteristics for $\beta < 0$, $\beta = 0$, $\beta > 0$ are shown in insets. 1. ○ — egg lecithin in n-heptane, KCl. 2. × — glycerolmonooleate in n-heptane, KCl. 3. ● — glycerolmonooleate in n-hexadecane, KCl. 4. △ — glycerolmonoolete in n-hexadecane, LiCl.

as the substitution of a non-polar lipid (glycerolmonooleate) for a polar one (egg lecithin), change in the electrolyte composition, change of the membrane solvent. It is therefore interesting to study the effect of these factors also on the non-linearity coefficient β. We have studied the effect of different electrolytes and membrane solvents on the β coefficient of monooleate membranes (curves 2—4, Fig. 9). There is a noteworthy agreement of all curves except the point $c = 0.1$ mol/l. Substitutions of K^+ for Li^+ (curves 3 and 4) and of n-heptane for n-hexadecane (curves 2 and 3) did not change the nature of the β dependence on the electrolyte concentration c.

Substitution of n-heptane for n-hexadecane resulted in a change of the membrane thickness from 4.8 nm to 3.1 nm (Hladky and Haydon 1972). In membranes prepared from glycorolmonooleate in n-hexadecane a phase transition takes place approximately at 15.5 °C at which the BLM thickness is changed 1.16 times (White 1974). We measured the value of β on similar membranes in 0.1 mol/l KCl at slow cooling several minutes and warming of BLM from 14 to 20 °C. The β coefficient did not change during the phase transition either.

The basic quantitative parameters of ionic channels have hitherto included life time τ and channel conductance λ. The non-linearity of channel VA has been studied only qualitatively (Urban et al. 1978). Quantitative results similar to those reported in our previous work (Flerov et al. 1981) were obtained only by Andersen

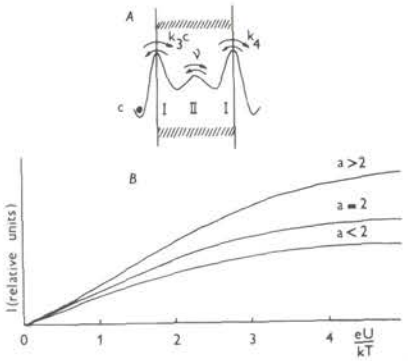

Fig. 10. Three potential-barrier model of gramicidin A ICH and its VA characteristics (from Markin and Chizmadzhev 1974). *A*: model of the channel, k_3c, k_4, ν — constants of the transfer rates over the barriers, c — concentration of the ions in the electrolyte. I — external barrier. II — internal barrier. *B*: VA characteristics of the model for different values of the parameter $a = \frac{k_3c + k_4}{2\nu}$.

(1983); he used rather difficult single-channel measurements at very high potencials (up to 500 mV). Our measurements of β with different membranes indicate that β is a new quantitative parameter of the channel; its changes due to membrane and electrolyte variations do not correlate with changes in τ and λ, respectively. Variations of the membrane thickness upon substitution of the solvent and phase transition strongly affect τ, leaving λ unchanged and having no influence on the value of β. Cation replacement in the electrolyte resulted in no change of τ, it strongly changed λ, while leaving β unchanged. At the same time, a replacement of the lipid changed the value of β but not that of λ. It may therefore be expected that the study of the coefficient of non-linearity of ionic channels offers new possibilities in the analysis of the mechanism of the ionic transport.

Comparison of experimental results and theoretical models of conductance of Gramicidin A channel

Our study of the non-linearity of VA of GRA channels allows to compare the experimentally measured parameters with the "forecasts" of the corresponding theoretical models. Models usually represent the potential cross section of the channel in form of holes and barriers. They assume that in each potential hole, a certain number of ions can be present (Sandblom et al. 1977).

Let us analyze a simple model of GRA channel given in the paper by Markin and Chizmadzhev (1974). In this model, the channel consisting of two monomers, shows three potential barriers and two holes (Fig. 10). It is assumed that maximum one ion can be presented in each potential hole and the rate of the ion jump over

the middle barrier is proportional only to the concentration of the ions in the neighbouring holes. The rate of the jumps over the marginal barriers inside the channel is proportional to the concentration of the penetrating ion in the electrolyte in the vicinity of the channel. For such a model, the volt-ampere characteristics is as follows:

$$i(U)=\frac{2z\ \mathrm{e}\nu k_3 k_4 c}{k_3 c+k_4}\cdot\frac{\mathrm{sh}(z\ \mathrm{e}U/2kT)}{k_3 c+k_4+2\nu\ \mathrm{ch}(zeU/2kT)}, \tag{19}$$

where ν, k_3, k_4 are the constants of the respective rates over the barriers, c is the concentration of the cation, z is the cation charge. Let $z=1$. We shall develop VA according to the U powers and we leave only the components up to U^3. Let $\frac{k_3 c+k_4}{2\nu}=a$ then:

$$\begin{aligned} i(U)&=\frac{\mathrm{e}k_3 k_4 c}{2\nu a}\cdot\frac{\mathrm{sh}(\mathrm{e}U/2kT)}{a+\mathrm{ch}(\mathrm{e}U/2\mathrm{k}T)}\approx \\ &\approx\frac{\mathrm{e}k_3 k_4 c(\mathrm{e}U/2\mathrm{k}T)}{2\nu a(1+a)}\cdot\left[1+\left(\frac{\mathrm{e}U}{2\mathrm{k}T}\right)^2\left(\frac{1}{6}-\frac{1}{2a+2}\right)\right] \end{aligned} \tag{20}$$

$$\lambda=\frac{\mathrm{e}k_3 k_4 c}{2\nu a(a+1)}\cdot\frac{\mathrm{e}}{2\mathrm{k}T} \tag{21}$$

$$\beta=\frac{1}{6}\cdot\frac{a-2}{a+1}\cdot\left(\frac{\mathrm{e}}{2\mathrm{k}T}\right)^2 \tag{22}$$

Consequently the coefficient β of VA non-linearity is associated with the kinetic constant and ions concentration (parameter a), respectively. We are going now to analyze the characteristic features of this model. From (22) and for $a<2$ we get $\beta<0$, i. e. VA is sublinear, and for $a>2$ we get $\beta>0$, i. e. VA is superlinear. Fig. 10 shows VA for different values of a. It is actually possible to follow the change of VA from a sublinear to a superlinear form when the parameter a increases from values lower than 2, to values higher than 2, however within a narrow interval of voltages not exceeding 50 mV. The theoretical VA reaches the constant level of VA at voltage values on the membrane between 150—200 mV. This is due to the fact that the argument of the functions sh and ch in the relationship (19) is $(\mathrm{e}U/2\mathrm{k}T)\approx$ U/50 mV. At the same time it is evident from the VA obtained by us (see Fig. 4*B*) that up to the voltage of at least 300 mV (and, in some experiments, even 400 mV) no signs of VA saturation could be observed.

As shown in Fig. 9 in the interval of cation concentrations from 0.03 to 3 mol/l the coefficient of non-linearity can be expressed by following relationship:

$$\beta(c)\approx 7\ \lg c,\quad [\mathrm{V}^{-2}] \tag{23}$$

It is not difficult to prove that the concentration dependence of (23) cannot be

approximated by the relationship (22) over the whole interval of the concentrations for none of the values of the parameters k_3, k_4 and v.

An analysis of other theoretical models of the GRA channel shows that neither of them can explain quantitatively the obtained dependence of β on cation concentration — (Fig. 9). E. g., in models where the potential profile of the channel is expressed in the form of a great number of equal barriers (Tredgold 1979) there is no dependence of β on electrolyte concentration. Moreover, one of the following facts remains unexplained:

a) no saturation is observed in VA characteristics of GRA channels, at least up to voltage values of 300 mV (Fig. 4*B*).
b) for small voltages (up to 100 mV) the VA non-linearity is rather remarkable, β can reach $-20\ V^{-2}$ (Fig. 9).

The above analysis is only illustrative, merely to show that the above model of the channel does not agree with experimental results. Thus, the suggestion concerning the association of β with kinetic constant does actually not hold. The above fact may possibly be explained by models considering Coulomb interactions among ions simultaneously present in the channel (see e. g. Markin and Chizmadzhev 1974).

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