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Photodynamic inactivation of gramicidin channels: a flash-photolysis study

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Abstract

Photosensitized inactivation of ionic channels formed by gramicidin in the planar bilayer lipid membrane (BLM) has been studied upon exposure of the BLM to single flashes of visible light in the presence of tetrasulphonated aluminium phthalocyanine. The gramicidin photoinactivation is inhibited by the addition of unsaturated phospholipids to the membrane-forming solution as well as by the addition of azide to the bathing solution, consistent with involvement of singlet oxygen. The characteristic time of the photoinactivation (τ) does not change markedly under these conditions. Moreover, τ remains nearly constant upon alteration of the flash energy and the photosensitizer concentration. The value of τ appears to be sensitive to the gramicidin concentration and to the factors affecting the open time of the gramicidin channels, namely the temperature and the solvent used in the membrane-forming solution. The photoinactivation is not observed with covalent gramicidin dimers. The equations derived from the model of Bamberg and Laeuger (J. Membrane Biol. (1973) 11, 177–194), describing the relaxation of the gramicidin-induced conductance after a sudden distortion of the dimer-monomer equilibrium, are shown to explain consistently the time course of the photoinactivation provided that the damage of the gramicidin molecules leads to deviation from the equilibrium.

Keywords: Planar bilayer lipid membrane; Ionic channel; Gramicidin; Photodynamic action; Phthalocyanine

1. Introduction

Numerous investigations of a photodynamic effect on cells and tissues of living organisms have shown that biological membranes represent an important target of the photodynamic action [1]. In particular, a series of research works has demonstrated photomodification of specific ionic currents in nerve, skeletal muscle and cardiac cells [2–6]. This photomodification is suggested to take place due to interaction of photosensitizer-generated reactive oxygen species with the proteins forming sodium, potassium and calcium channels [2] (see refs. therein). Photosensitized inactivation of channels formed by pentadecapeptide gramicidin A in artificial bilayer lipid membranes may serve as a model for studying the mechanism of the photodynamic modification of natural ionic channels. This system was used to investigate inactivation of ionic channels induced by ultraviolet radiation. It was shown that ultraviolet inactivation of both natural [7,8] and model (gramicidin) [9–11] channels is associated with modification of tryptophan residues.

It has been shown recently that visible light irradiation of bilayer lipid membranes in the presence of a photosensitizer leads to suppression of gramicidin-induced conductance [12,13]. The decrease in the conductance is due preferentially to the reduction of the number of open channels. In this paper we present the results of studying the mechanism of the light-induced inactivation of gramicidin channels in a bilayer lipid membrane (BLM) by exciting the BLM with single flashes of visible light in the presence of an effective sensitizer of singlet oxygen generation, phthalocyanine.

2. Materials and methods

BLMs are formed from a solution of 2% diphytanoylphosphatidylcholine (Avanti Polar Lipids) in *n*-decane (unless otherwise stated) by the brush technique [14]

Abbreviations: BLM, bilayer lipid membrane; $AlPcS_4$, tetrasulphonated aluminium phthalocyanine.

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on a 0.55-mm diameter hole in a Teflon partition separating two aqueous compartments. The bathing aqueous solutions in the cell contain 1 M KCl, 10 mM MES, 10 mM Tris, 10 mM β-alanine. Gramicidin (Sigma) is added from a stock solution in ethanol to the bathing solutions at both sides of the BLM and routinely incubated for 15 min at constant stirring. Succinyl-*bis*-gramicidin was a kind gift of Prof. A.S. Arseniev (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow). Aluminium tetrasulphophthalocyanine ($AlPcS_4$) was kindly provided by Dr. M.G. Galpern (Research Institute of Organic Intermediates and Dyes, Moscow). $AlPcS_4$ is added to the bathing solution at the *trans* side (the *cis*-side is the front side with respect to the flash lamp). Asolectin (L-α-phosphatidylcholine, type IV-S from soybeans) and cardiolipin were purchased from Sigma.

The electric current (I) is recorded under voltage-clamp conditions. The currents are measured by means of a K544UD1A operational amplifier (Russia), an analog of μA740 (Fairchild, USA), digitized by DT2801A (Data Translation, USA). In most experiments the data sampling interval is set to 20–50 ms. Ag-AgCl electrodes are put directly into the cell, the potential (U) of an amplitude of 65 mV is applied to the BLM. Computer analysis is carried out using the SCAN program, supplied generously by J. Dempster (Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK).

BLMs are illuminated by single flashes produced by a xenon lamp with flash energy of about 0.3 J and flash duration ≤ 3 ms. The glass filter cutting off the light with the wavelengths < 500 nm is placed in front of the flash lamp.

3. Results

Fig. 1 illustrates the time course of the current through the BLM. It is seen that excitation of BLM with a flash of visible light in the presence of tetrasulphonated aluminium phthalocyanine leads to a decrease in the gramicidin-mediated current. The time course of the current decrease fits well a monoexponential curve with an exponential factor τ (below called the characteristic time) of ca. 1 s. Inhibition of the conductance λ ($\lambda = I/(U \cdot S)$, where S is the area of the BLM) is not observed upon exposure of the BLM to the flash in the absence of a photosensitizer. The value of τ exceeds by several orders of magnitude the values of the lifetime of singlet oxygen [15] and oxygen-containing radicals [16]. Surprisingly, the time constant of the decrease in the current appears to be similar to the value of the lifetime of the open state of a gramicidin channel [17]. In this connection it can be proposed that the characteristic time of the photosensitized inactivation of gramicidin does not depend on the concentration of reactive oxygen species generated by a photosensitizer but changes with an alteration in parameters of a gramicidin channel. In fact, the τ value does not change noticeably upon the diminution of the flash energy (Fig. 2) as well as upon the alteration of the phthalocyanine concentration (Fig. 3). The relative

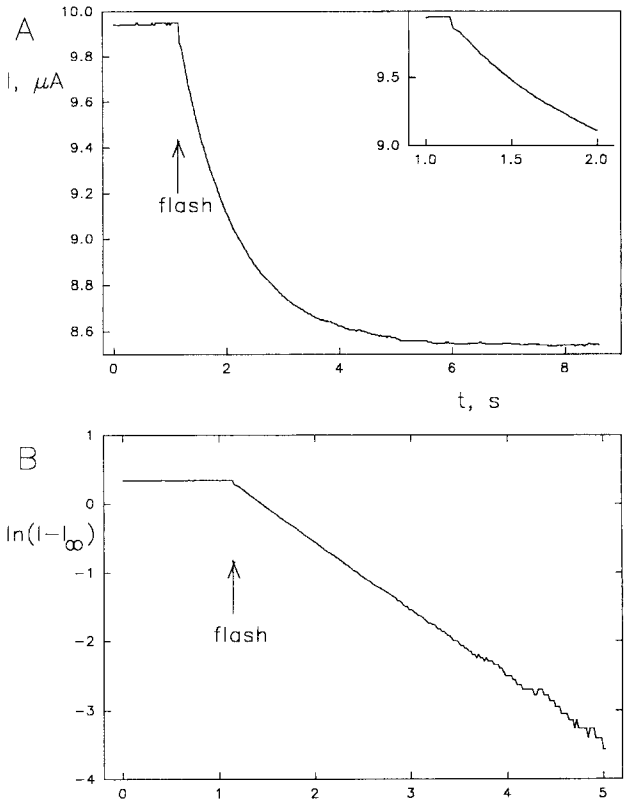

Fig. 1. A: Time course of the effect of the visible light flash on the gramicidin-mediated current (I) through BLM in the presence of 1 μM $AlPcS_4$. The pH of the bathing solution is 7.0, $T = 26$°C. Inset: the same data in a different time scale. B: The same data presented as $\ln(I - I_\infty)$ versus time, t (s).

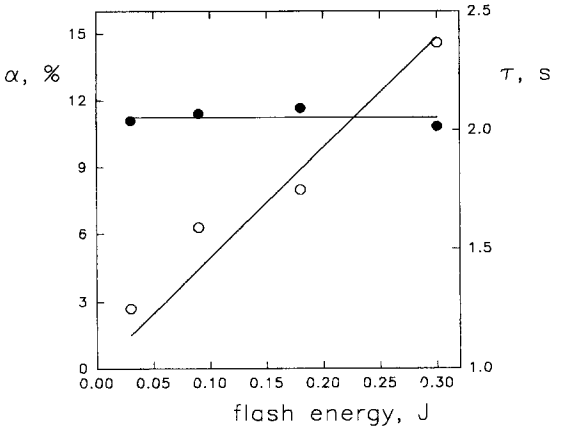

Fig. 2. Dependence of the relative amplitude (α, open circles) and the characteristic time (τ, closed circles) of the light-induced decrease in the gramicidin-mediated current through BLM on the flash energy in the presence of 1 μM phthalocyanine. The pH of the bathing solution is 3.0, $T = 18$°C. The initial current is 2.7 μA. The flash energy is gradually diminished by glass filters.

amplitude, $\alpha = (I_0 - I_\infty)/I_0$ (where I_0 is the value of the initial current and I_∞ is the value of the stationary current), of the light-induced decrease in the gramicidin-mediated current grows upon increasing both light intensity and the photosensitizer concentration. It is worth noting that there is a maximum in the concentration dependence of α. At phthalocyanine concentrations higher than 10^{-6} M, α begins to drop, probably due to aggregation of the photosensitizer at the membrane-water interface. It has been shown that tetrasulphonated aluminium phthalocyanine is not susceptible to aggregation in water [18]. With rose bengal used as a photosensitizer, the suppression of the gramicidin-mediated current is observed having a τ value similar to that in the presence of phthalocyanine. The data on the effect of scavengers of reactive oxygen species are also in agreement with our proposal. Fig. 4 shows that α drops upon increasing the concentration of sodium azide in the bathing solution. The characteristic time of the photoinactivation does not change noticeably. Similar results are obtained with 0.5% α-tocopherol (not shown). An addition of superoxide dismutase (300 Unit/ml) does not produce any effect on the time course of the current decrease (not shown). The data on the effect of sodium azide support the participation of singlet oxygen in the photosensitized inactivation of gramicidin. In line with this are the data on the effect of the BLM lipid composition on the gramicidin inactivation. Upon addition of cardiolipin (containing linoleoyl residues with two double bonds) to the membrane-forming solution of diphytanoylphosphatidylcholine (10:90, w:w) the amplitude of the photoinactivation decreases about 20 times, but the characteristic time remains unchanged. When asolectin containing multiple unsaturated hydrocarbon chains is used instead of

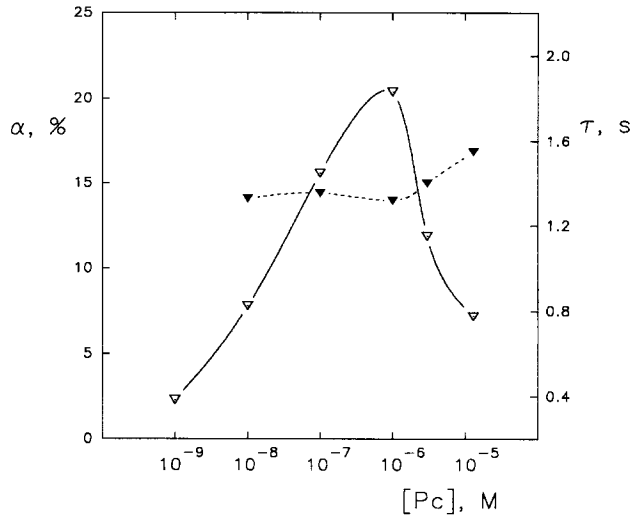

Fig. 3. Dependence of the relative amplitude (α, open triangles) and the characteristic time (τ, closed triangles) of the light-induced decrease in the gramicidin-mediated current on the concentration of phthalocyanine. The pH of the bathing solution is 3.0, $T = 18$°C. The initial current is 1.0 μA.

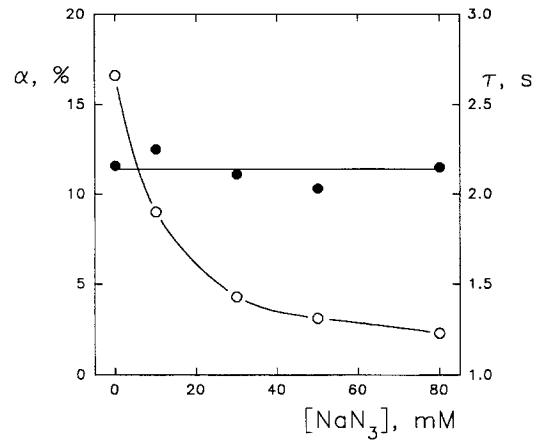

Fig. 4. Dependence of the relative amplitude (α, open circles) and the characteristic time (τ, closed circles) of the light-induced decrease in the gramicidin-mediated current on the concentration of sodium azide (NaN_3) in the bathing solution in the presence of 1 μM $AlPcS_4$. The pH of the bathing solution is 4.2, $T = 18$°C. The initial current is 0.6 μA.

diphytanoylphosphatidylcholine having no double bonds, photoinactivation is hardly detectable. Obviously, the double bonds of unsaturated fatty acid residues in cardiolipin and asolectin play the role of scavengers of reactive oxygen species, thereby protecting gramicidin channels from photoinactivation.

It is interesting to examine the influence of factors which modify the characteristics of the gramicidin-induced conductance on the parameters of its photoinactivation. The data presented in Fig. 5 show that the rise in the stationary BLM conductance as a result of the increase in the gramicidin concentration is accompanied by a considerable shortening of the characteristic time of photoinactivation and a marked reduction of its relative amplitude, whereas the absolute value of the amplitude of photoinactivation increases.

It can be supposed that the rate of photoinactivation changes with variation in the open time of the gramicidin channel. It is known that the latter depends on the solvent used in the membrane-forming solution [19]. In particular, it is considerably higher in the presence of squalane instead of *n*-decane [20]. As seen from Fig. 6, the kinetics of photoinactivation is significantly slowed in the presence of squalane (compared to *n*-decane) in the membrane-forming solution. However, in this case a fast phase of photoinactivation appears. The ratio of the amplitudes of the fast and slow components of the current decrease grows with an increase in the stationary conductance (Fig. 7). In the absence of squalane the fast component is hardly discernible (see inset in Fig. 1).

It is known from the literature [21,22] that the open time of the channels formed by the covalent dimers of gramicidin exceeds the corresponding value for the monomer gramicidin channels by approximately two orders of magnitude. Our experiments with covalently dimerized gramicidin A, succinyl-*bis*-gramicidin, have revealed

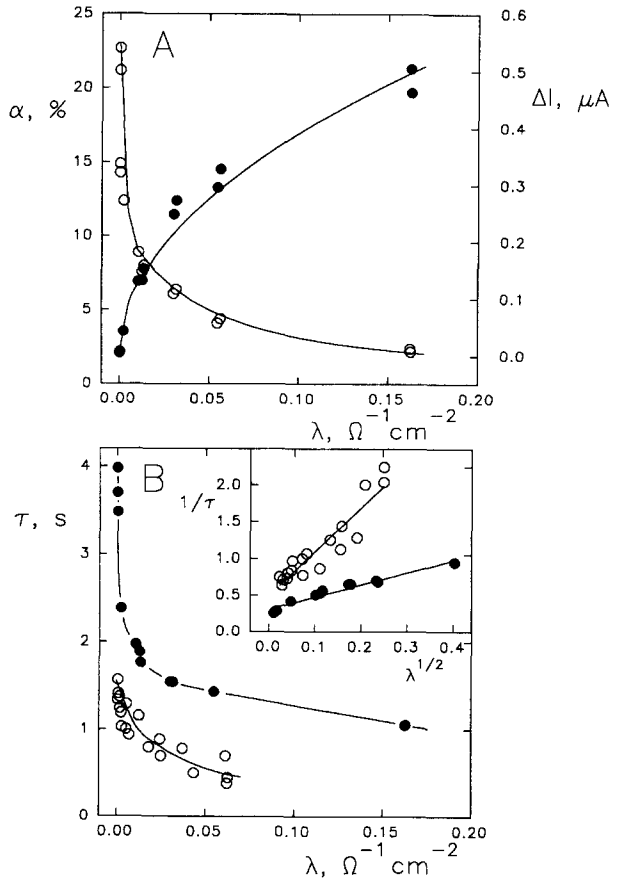

Fig. 5. A: The dependence of the relative amplitude (α, open circles) and the absolute amplitude ($\Delta I = I_0 - I_\infty$, closed circles) of the light-induced decrease in the gramicidin-mediated current on the BLM conductance (λ) in the presence of 1 μM $AlPcS_4$. The pH of the bathing solution is 3.0, $T = 18°C$. B: Dependence of the characteristic time (τ) of the light-induced decrease in the gramicidin-mediated current on the BLM conductance (λ) in the presence of 1 μM $AlPcS_4$ at pH 3.0, $T = 18°C$ (closed circles) and at pH 7.0, $T = 26°C$ (open circles). Inset: the reciprocal characteristic time τ^{-1} is plotted versus the square root of λ for the two cases indicated. Estimations of the dissociation and association rate constants from these data give the following values: $K_d = 0.3\ s^{-1}$, $K_R = 5.6 \cdot 10^{12}\ s^{-1}\ mol^{-1}\ cm^2$ at 18°C and $K_d = 0.48\ s^{-1}$, $K_R = 4.6 \cdot 10^{13}\ s^{-1}\ mol^{-1}\ cm^2$ at 26°C.

that photoinactivation (slow component) is not observed in this case (data not shown). Only the fast component of a very small amplitude is detected.

According to the data of Refs. [17,23], the open time of the gramicidin channels depends essentially on temperature: it decreases with increasing temperature. Thus it is important to study the dependence of the rate of the channel photoinactivation on temperature. The results presented in Fig. 8 show that the photosensitized inactivation is accelerated with increase in the temperature. Using the Arrhenius plot, it appears that ln (τ^{-1}) depends linearly on T^{-1} with an activation energy of about 22 kcal/mol.

Since photosensitized modification of natural ionic channels has been shown to depend on pH [5], it is interesting to study the pH dependence of gramicidin channel photoinactivation. Our experiments have shown

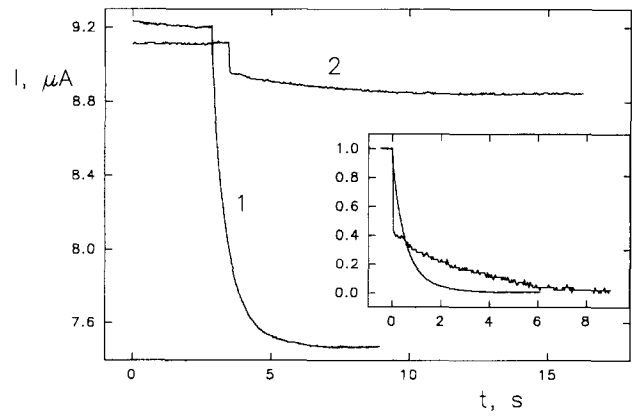

Fig. 6. Effect of the visible light flash on the gramicidin-mediated current through BLM in the presence of 1 μM $AlPcS_4$. (1) BLM formed from the *n*-decane solution of 2% diphytanoylphosphatidylcholine and 1% cholesterol. (2) BLM formed from the squalane solution of 2% diphytanoylphosphatidylcholine and 1% cholesterol. The pH of the bathing solution is 7.0, $T = 26°C$. Inset: the normalized curves (1 and 2) of the light-induced decrease in the current.

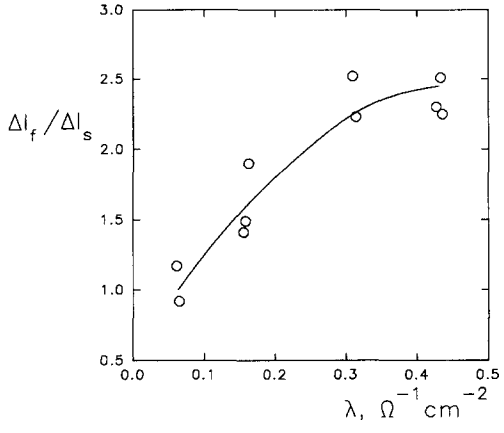

Fig. 7. Dependence of the ratio ($\Delta I_f / \Delta I_s$) of the amplitudes of the fast and slow phases of the current decrease on the BLM conductance (λ). The BLM is formed from the squalane solution of 2% diphytanoylphosphatidylcholine and 1% cholesterol. The pH of the bathing solution is 7.0, $T = 21°C$.

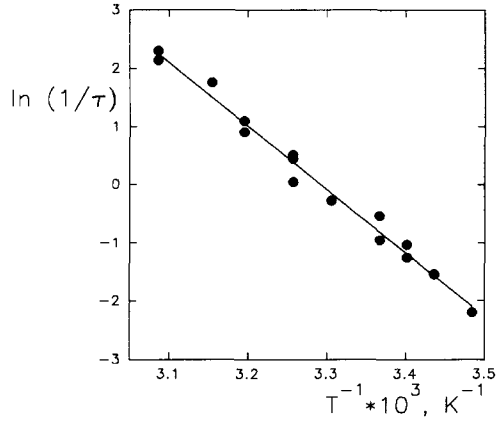

Fig. 8. Dependence of ln(1/τ) on the temperature (*T*) of the bathing solution (pH 3.0). The initial current through BLM at each temperature is 1.3 μA.

that the photoinactivation parameters are independent of pH in the range of 3–9 (data not shown).

4. Discussion

Based on the lower photosensitivity of the channels formed by gramicidin with a reduced number of tryptophan residues [12], it was suggested that the photodynamic inactivation of gramicidin is caused by modification of tryptophan residues as is the case with inactivation induced by ionizing [24–26] or ultraviolet [8,9,25,27] radiation. The tryptophan residues tending to form hydrogen bonds with polar groups near the lipid head-group region and consequently located near the hydrophobic–hydrophilic interface in lipid bilayers are believed to be crucial for maintaining the structure and conductivity of the gramicidin channel [28,29]. It can be supposed that modification of gramicidin due to photosensitized damage of tryptophan residues leads to perturbation of the dimer-monomer equilibrium.

In order to explain the results of examination of the kinetics of the photodynamic inactivation of gramicidin presented here, one should address the study of Bamberg and Laeuger [30], in which the authors considered an equilibrium between monomers (M) and dimers (D) of gramicidin in the planar bilayer lipid membrane:

$$M + M \underset{K_d}{\overset{K_R}{\rightleftarrows}} D$$

The time course of equilibration is shown to be determined by the following parameters: the initial concentration of gramicidin (N), the gramicidin dissociation constant (K_d) and the equilibrium constant (K) for association (dimerization) of gramicidin in the membrane (see Eq. 10 in Ref. [30]) which in turn depends on such factors as electrical field intensity, temperature, pressure, etc.:

$$\tau = K_d^{-1}(1 + 8NK)^{-1/2}$$

It is generally accepted that the current through the membrane containing gramicidin is directly proportional to the concentration of the gramicidin dimers [17,30]. Thus the kinetics of the changes in the current upon photoinactivation reflects the time course of variation of the gramicidin dimer concentration. Inactivation of both dimers and monomers as a result of interaction with singlet oxygen generated upon excitation of the photosensitizer leads to deviation of the system from the equilibrium. Direct inactivation of gramicidin dimers should manifest itself in an immediate drop of the transmembrane current (in the time range under study), after which the transition of the system to a new equilibrium state is expected to occur. In contrast, in the case of inactivation of the monomers the time course of the change in the current reflects only the process of the dimer-monomer equilibration. In general, the kinetics of the current changes may have both fast and slow components. The ratio of the amplitudes of these components

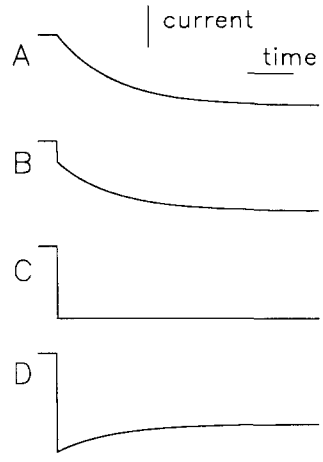

Fig. 9. Theoretical curves simulating the time courses of the gramicidin-mediated current. The curves are calculated from Eq. 12 from [28], describing the time dependence of the concentration of the gramicidin dimers after a sudden distortion of the dimer-monomer equilibrium. Under our experimental conditions, deviation from the equilibrium is caused by a change in the concentration of monomers M and/or dimers D due to their inactivation by the light flash in the presence of a photosensitizer. It is assumed that: A, only monomers are inactivated; B, both monomers and dimers are inactivated so that immediately after inactivation their concentrations ([M] and [D], respectively) correspond to $[D]/[M]^2 > K$; C, the same as (B) but at $[D]/[M]^2 = K$; D, the same as (B) but at $[D]/[M]^2 < K$.

may be of different sign and magnitude depending on the gramicidin concentration, the equilibrium constant of the gramicidin dimerization and the ratio of the monomer and dimer photosensitivities. Fig. 9 illustrates particular cases of the kinetics of the current changes. The theoretical curves presented in this figure were calculated from Eq. 12 from [30]. Our experimental data obviously correspond to curve A of Fig. 9 with a negligibly small portion of the fast phase (see Fig. 1). If squalane is present in the membrane-forming solution (Fig. 6), the fast component of the current decrease is well discernible. This case corresponds to curve B. The fast component is most likely due to inactivation of the gramicidin dimers which is favored by growing of the portion of the fast component with an increase in the stationary conductance (Fig. 7). In fact from Eqs. 2 and 3 describing the dimer-monomer equilibrium in Ref. [30], it can easily be derived that the portion of the gramicidin dimers in the overall number of gramicidin molecules grows with an increase in the conductance.

Thus the slow decline of the gramicidin-induced BLM conductance described in this paper can be attributed to the process of equilibration of the system after distortion of the equilibrium as a result of inactivation of the monomers by the light flash (apparently via modification of tryptophan residues). This conclusion is compatible with the results obtained by ultraviolet flash photolysis of gramicidin-doped lipid bilayers [27]. Busath and Hayon [27] also proposed that a relaxation of dimer-monomer equilibrium perturbed by inactivation manifests itself in slow kinetics of gramicidin channel conductance following an initial

abrupt flash-induced conductance decay. In contrast, Straessle et al. [24] observed the dose-dependent kinetics of ionizing radiation inactivation of gramicidin channels, indicating that a long-lived radical participates in the process of inactivation. In our opinion this evidence is in favour of different mechanisms of inactivation induced by photosensitization and ionizing radiation. Recently Tarr et al. [31] presented data on the progression of cardiac potassium current modification after brief exposure to illumination in the presence of rose bengal. The authors concluded that the progression resulted most probably from the kinetics of potassium channel state transitions.

By plotting the reciprocal characteristic time (τ^{-1}) as a function of the square root of the stationary conductance λ (Fig. 5B, inset) according to Eq. (16) from [30]:

$$\tau = \left[K_d + 4\left(K_d K_R \lambda / N^o \Lambda \right)^{1/2} \right]^{-1}$$

where N^o is Avogadro's number and Λ is the conductance of the single channel, we have estimated the rate constants of association K_R and dissociation K_d and the equilibrium constant K of the dimerization reaction of gramicidin A. The value of K ($9.5 \cdot 10^{13}$ $mol^{-1} \cdot cm^2$) calculated from the experimental data obtained at 26°C (at 65 mV) is consistent with the literature data: $K = 12 \cdot 10^{13}$ $mol^{-1} \cdot cm^2$ at 25°C at $U = 135$ mV in [23] and $21 \cdot 10^{13}$ $mol^{-1} \cdot cm^2$ at 25°C at $U = 60$ mV in Ref. [32]. The data on variation of characteristic time of photoinactivation with temperature and solvent, as well as the linear dependence of the rate of photoinactivation on the square root of the conductance, support our conclusion about the nature of the slow time course of photoinactivation, namely these kinetics reflect the dimer–monomer equilibration of gramicidin.

Acknowledgements

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