Biochimica et Biophysica Acta, 980 (1989) 305–314
Elsevier

BBA 74393

Radiolysis and photolysis of ion channels formed by analogues of gramicidin A with a varying number of tryptophan residues

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(Received 13 December 1988)

Key words: Ion channel; Gramicidin A; Radiolysis; Photolysis; Lipid membrane

The ion channels formed by gramicidin A in planar lipid membranes may be inactivated by application of comparatively small doses of ionizing radiation (radiolysis) or by UV irradiation (photolysis). Both effects are reduced by several orders of magnitude, if the four tryptophan residues of gramicidin A are replaced by phenylalanines, tyrosines or by naphthylalanines. The present communication reports on the influence of a varying number of tryptophan residues per gramicidin monomer. The sensitivity of the channels towards photolysis was found to be roughly proportional to the number of tryptophans. The channels show complete inactivation in the presence of only one tryptophan residue per monomer. In the case of radiolysis, complete inactivation was observed for normal gramicidin A only. For those analogues having only one (or two) tryptophan residues per monomer, part of the initial conductance was found to be insensitive towards irradiation. As only small changes of the single-channel characteristics (amplitude and life-time) were observed, the radiation-sensitive part was mainly attributed to a reduced rate of channel formation. It is interpreted as a radiation-induced inhibition of the aggregation of gramicidin dimers. Aggregation, favoured by Trp-Trp contacts, is thought to represent an essential step for channel opening.

Introduction

The conductance of planar lipid membranes in the presence of ion channels formed by gramicidin A has been found to decrease by many orders of magnitude on exposing the membrane either to UV light (photolysis) or to ionizing radiation (radiolysis) [1–4]. Both effects have been shown to be due to the presence of tryptophan residues. While photolysis may be considered as a direct radiation effect initiated by light absorption of the tryptophan chromophores [1,2], radiolysis is an indirect radiation effect caused by radiation-induced water radicals [3,4]. Experiments performed in the presence of different radical scavengers [4] have led to the conclusion that channel inactivation by radiolysis is due to a subsequent reaction of OH and HO_2 radicals with the tryptophan residues of gramicidin A.

Our previous study was confined to normal gramicidin A. There are four different tryptophan residues of this pentadecapeptide which, according to Urry's [5,6] helical model of dimeric channel structure, are presumed to be in close contact with the aqueous phase. The present communication is aimed at the role of the four tryptophan residues. Analogues of valine-gramicidin A with a different number of tryptophan residues were synthesized and were compared with respect to their channel inactivation by radiolysis and photolysis. Characteristic differences between the two kinds of inactivation were observed, which allow further insight into the mechanism of formation of open gramicidin channels.

Materials and Methods

The structures of the different analogues used throughout the present study are illustrated in Fig. 1. The tryptophan residues of normal gramicidin A are replaced either by tyrosines (analogue GT), *O*-benzyl-L-tyrosines (analogue GT(Bzl)), or by naphthylalanines (analogues GN, $GN^{9,11,15}$, $GN^{11,13}$ and $GN^{9,15}$). The analogues were prepared as described by Trudelle and Heitz [7] and by Ranjalahy-Rasoloarijao et al. [8].

In addition to the analogues mentioned so far, the study of the analogue GM^- which has the four tryp-

Abbreviations of the analogues of gramicidin A (GA) investigated are presented in Fig. 1, p. 306.

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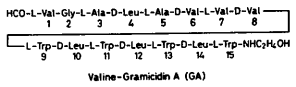

Fig. 1. Structure and abbreviations of the different analogues investigated throughout the present study.

GT: [$Tyr^{9,11,13,15}$]GA	(Tyr ≙ L-tyrosine)
GT(Bzl): [$BzTyr^{9,11,13,15}$]GA	(T(Bzl) ≙ BzTyr ≙ *O*-benzyl-L-tyrosine)
GN: [$Nap^{9,11,13,15}$]GA	(N ≙ Nap ≙ L-naphthylalanine)
$GN^{9,15}$: [$Nap^{9,15}$]GA;	$GN^{11,13}$: [$Nap^{11,13}$]GA
$GN^{9,11,15}$: [$Nap^{9,11,15}$]GA;	

tophan residues replaced by phenylalanines, was continued [9,4].

Radiolysis was investigated using either a conventional X-ray source (Siemens Stabilipan) or pulses of 14 MeV electrons delivered from the linear accelerator (Linac) of the Hahn-Meitner-Institut. Details of these experiments have been described previously [4].

Photolysis experiments were performed using UV light from a xenon lamp (Osram XBO 150W/1) focused onto the membrane. The light was passed through an infrared water filter and through filter UG11/1mm (Schott) to adapt the wavelength spectrum to the absorption spectrum of tryptophan (250–300 nm). The diameter of the membranes (1–3 mm) was small enough to provide for a homogeneous illumination. A mechanical shutter was used to control the time of illumination.

For further details of the experiments (both materials and methods) the reader should consult our previous publication on this topic [4]. All experiments were performed at room temperature (19 ± 1°C).

Results

The strong influence of the number of tryptophan residues on the sensitivity of gramicidin channels towards radiolysis or photolysis is demonstrated by experiments illustrated in Figs. 2–5. Fig. 2 indicates that the channels formed by the analogue $GN^{9,11,15}$, which has only one tryptophan residue per monomer, are hardly influenced by X-rays within a dose range of 1–10 Gy. Application of such a dose, on the other hand, gives rise to an almost complete inactivation of channels formed by normal gramicidin A.

To study radiolysis of $GN^{9,11,15}$ larger doses must be applied. To keep the exposure time in the range of several minutes, such experiments were performed at the linear accelerator (Linac) of the Hahn-Meitner-Institut, supplying 5–100-ns pulses of 14 MeV electrons of sufficiently high intensity. The membranes were irradiated by a sequence of pulses (repetition time 40 ms). The effect on the membrane conductance is represented in

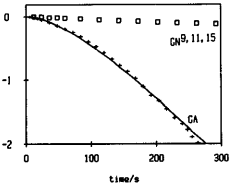

Fig. 2. Radiation inactivation by 220 kV X-rays. The membranes (dioleoylphosphatidylcholine/*n*-decane) were formed in air-saturated aqueous solutions of 1 M NaCl (pH 3, unbuffered) containing trace amounts of gramicidin A (+) or $GN^{9,11,15}$ (□). The membrane current, *I*, at a constant voltage of 60 mV was measured as a function of time, while the membranes were exposed to a constant dose rate of 2.9 Gy/min. The initial current, I_o, before irradiation was 1.5 μA (GA) and 5.6 nA ($GN^{9,11,15}$) (membrane area $5 \cdot 10^{-3}$ cm^2). The full line represents a fit to Eqn. 14 of Ref. 4.

Fig. 3. For membranes formed from dioleoylphosphatidylcholine in the presence of $GN^{9,11,15}$ application of a radiation dose as large as 150 Gy gives rise to only a comparatively small reduction (about 50%) of the membrane conductance. Further irradiation (up to at least 600 Gy) does not lead to any further decrease of the conductance, i.e. only the part $I_o - I_1$ of the current may be influenced by ionizing radiation, where I_1 represents the virtually radiation-insensitive current observed at large doses (state 1).

The introduction of a second tryptophan residue (analogues $GN^{9,15}$ and $GN^{11,13}$) leads to a considerable increase of the radiation-sensitive fraction $\theta = (I_o - I_1)/I_o$ of the membrane conductance ($\theta \geq 98\%$). In the case of normal gramicidin A, with four tryptophan residues per monomer, I_1 is at least 4 orders of magnitude smaller than I_o. The final level, I_1, observed for gramicidin A in Figs. 3 and 4 is of the order determined

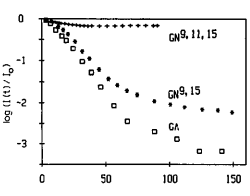

Fig. 3. Radiation inactivation by pulses of 14 MeV electrons. The experiments, apart from the radiation source, were performed under identical conditions to those described in the legend to Fig. 2. The initial conductance, λ_o, was between $1 \cdot 10^{-4}$ and $5 \cdot 10^{-4}$ S/cm^2 in each case. The scale of the dose is 1 Gy/unit (GA and $GN^{9,15}$) and 10 Gy/unit ($GN^{9,11,15}$), respectively.

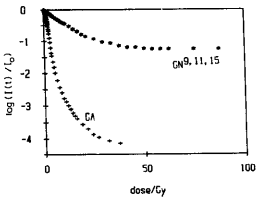

Fig. 4. Radiation inactivation by pulses of 14 MeV electrons. The experimental conditions were identical to those described in the legends to Figs. 2 and 3, except that the membranes were formed from diphytanoylphosphatidylcholine/*n*-decane. The initial conductance, λ_o, was $1.6 \cdot 10^{-2}$ S/cm^2 (GA) and $6 \cdot 10^{-5}$ S/cm^2 (GN^{9,11,15}).

by the basic conductance of the unmodified (pure) bilayer. At high initial conductance of the membrane ($\lambda \approx 0.1$ S/cm^2), a decrease of the current by up to 6 orders of magnitude was observed. In general, the magnitude of the decrease is determined only by the ratio of the initial conductance to the basic conductance of the pure bilayer.

The sample of gramicidin A used throughout the present study was of natural origin, i.e., it was a mixture of the three analogues gramicidin A, B, and C with the approximate ratio 8 : 1 : 2. The analogues B and C contain a phenylalanine (B) or a tyrosine (C) in position 11 (instead of a tryptophan). Therefore, 20–30% of the gramicidin molecules have only three Trp residues per monomer. The virtually complete inactivation, found in the presence of normal GA, allows the conclusion that three Trp residues are sufficient for the channel closure.

The results shown in Fig. 4 were obtained with membranes formed from diphytanoylphosphatidylcholine. The radiation-sensitive part, θ, of the conductance, in the presence of the analogue GN^{9,11,15} was found to be considerably larger ($\theta \approx 95\%$) as compared to membranes formed from dioleoylphosphatidylcholine ($\theta \approx 50\%$). This indicates an influence of the lipid structure on the radiolysis of the channels.

One may ask for the radiation dose, D_{37}, necessary to reduce the radiation-sensitive part, $I_o - I_1$, to 37% of its initial value. As summarized in Table I, the D_{37} dose was found to be rather similar for the analogues GA, GN^{9,15}, and GN^{11,13}. In the presence of only one Trp residue (analogue GN^{9,11,15}), however the sensitivity is decreased by a factor of 8.

Photolysis of gramicidin A and of the analogue GN^{9,11,15} is illustrated in Fig. 5. It was found that the current, at a continuous illumination of the membrane, could be fitted by a sum of two exponential terms according to

$$I(t)/I_o = \alpha \exp(-t/\tau_1) + (1-\alpha)\exp(-t/\tau_2) \qquad (1)$$

TABLE I

Relative values of the radiation dose D_{37} for channel inactivation by 14 MeV electrons

The D_{37} value is defined as the radiation dose necessary to reduce the difference $I_o - I_1$ to 37% of its initial value (see text). The value obtained for normal GA is 5 ± 0.9 Gy (mean $\pm$ S.D.). The values (mean of five different membranes) hold for membranes formed from dioleoylphosphatidylcholine (for further experimental details see legend to Fig. 2). In the case of analogue GN, no conductance decrease was observed up to doses $\leq$ 3000 GY. A similar behaviour was also found for GT(Bzl) and GM$^-$.

Analogue	Tryptophans/monomer	D_{37}(X)/D_{37} (GA)
GA	4	1
GN^{9,15}	2	1
GN^{11,13}	2	1.2
GN^{9,11,15}	1	8
GN	0	–
GT	0	50
GT(Bzl)	0	–
GM$^-$	0	–

Eqn. 1 implicates that photolysis, contrary to radiolysis, leads to complete inactivation of the channels. This is supported by the experimental data, though in the case of GN^{9,11,15} due to the long period of the process, inactivation was followed only to the 1% level of the initial conductance. There is, however, a further continuous decrease of conductance indicating that the final value is far below that level. In the case of normal gramicidin A, due to its higher sensitivity (see below), the initial membrane conductance was found to decrease by up to 6 orders of magnitude.

A further difference to radiolysis consists in the dependence of the radiation sensitivity on the number of tryptophan residues (cf. Table II). The sensitivity

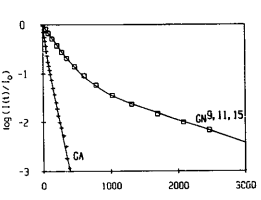

Fig. 5. Radiation inactivation by UV light. The membranes (diphytanoylphosphatidylcholine/*n*-decane) were formed in air-saturated aqueous solutions of 1 M NaCl (pH 3, unbuffered) containing trace amounts of gramicidin A (+) or GN^{9,11,15} (□). The membrane current, I, at a constant voltage of 60 mV was measured as a function of time, while the membranes were exposed to a constant intensity of UV light. The initial conductance, λ_o, before irradiation was $4 \cdot 10^{-5}$ S/cm^2 (GA) and $1.7 \cdot 10^{-5}$ S/cm^2 (GN^{9,11,15}) (membrane area $7 \cdot 10^{-2}$ cm^2). The full line represents a fit to Eqn. 1.

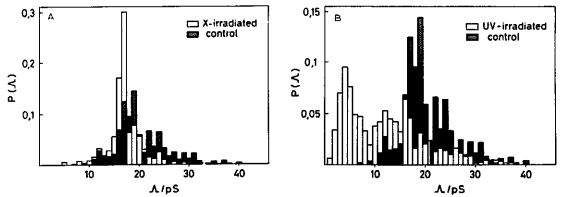

Fig. 6. Probability $P(\Lambda)$ of the observation of a conductance fluctuation Λ in the presence of single channels of the gramicidin analogue $GN^{9,11,15}$ before and after exposure to X-rays (A) or to UV light (B). The membranes were formed from monoolein-*n*-decane in the presence of 1 M CsCl. (A) A nominally 10^{-6} M solution of $GN^{9,11,15}$ in 1 M CsCl (pH 3) was irradiated with 2000 Gy, a radiation dose sufficient to convert virtually all $GN^{9,11,15}$ to state 1 (see Figs. 3 or 4). A small amount of this solution was added to the aqueous solutions surrounding the membrane and the resulting fluctuations were observed as a function of time at a constant voltage of 150 mV. The total number of analyzed channels was 928 (nonirradiated) and 894 (irradiated). The mean value, $\overline{\Lambda}$, of conductance fluctuations was 20.9 pS (control) and 17.3 pS (irradiated). (B) A (nominally) 10^{-5} M solution of $GN^{9,11,15}$ in 1 M CsCl was UV-irradiated for a time interval sufficient to reduce the macroscopic conductance (according to Fig. 5) to less than 1%. A small amount of this solution was added to the aqueous solutions surrounding the membrane and the resulting fluctuations were observed as a function of time at a constant voltage of 150 mV. The total number of analyzed channels was 928 (nonirradiated) and 1012 (irradiated). The mean value, $\overline{\Lambda}$, of conductance fluctuations was 20.9 pS (control) and 11.8 pS (irradiated).

(expressed either by the values of the characteristic time constants τ_1 and τ_2 or by the time t_{37} needed to reduce the current, I, to 37% of the initial value, I_o) is roughly proportional to the number of tryptophan residues per monomer.

The different behaviour of the channels towards radiolysis and photolysis is also apparent at the level of the single channel. Previous studies have shown that the histogram of the amplitudes of conductance fluctuations as well as the mean life-time of the channels in the open state are virtually identical before and after radiolysis of normal gramicidin A [4]. This indicates that radiolysis of normal GA is an 'all-or-nothing process', i.e. on the microscopic level only those channels contribute to the conductance, which are not affected by radiation. Photolysis, on the other hand, was shown to produce low conductance states of the channel which, on further exposure to UV light, show transitions to even smaller values [1].

TABLE II

Inactivation parameters found for photolysis of gramicidin A and its analogues

The data (mean values ± S.D. of five membranes) refer to membranes formed from diphytanoylphosphatidylcholine/*n*-decane (see legend to Fig. 5). The time t_{37}(GA) necessary to reduce the initial current in the presence of GA to 37% was 42.2 ± 7.5 s. The parameters α, τ_1 and τ_2 correspond to those in Eqn. 1. In the case of GT, GT(Bzl) and GN, no significant decrease of the current during illumination was observed.

Analogue	α	τ_1 (s)	τ_2 (s)	$t_{37}(X)/t_{37}$ (GA)
GA	0.45 ± 0.24	20.7 ± 13.3	61 ± 9	1
$GN^{9,15}$	0.48 ± 0.21	50.4 ± 17	129 ± 43	2.3
$GN^{11,13}$	0.83 ± 0.09	84.2 ± 24.4	206 ± 48	2
$GN^{9,11,15}$	0.53 ± 0.26	142 ± 47	397 ± 200	5.9

Previous experiments were extended by studying the behaviour of those gramicidin analogues which have only one or two tryptophan residues per monomer. Fig. 6B illustrates that the pronounced shift to smaller values of the distribution of single-channel amplitudes after partial photolysis is also observed in the case of $GN^{9,11,15}$. This indicates that the phenomenon (including the broadening of the distribution) is due to a light-induced modification of a single tryptophan residue and is not generated by a modification of a different number of tryptophan residues. Radiolysis, on the other hand, even after complete conversion to the state 1 observed at very large radiation doses ($D = 2000$

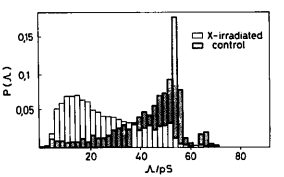

Fig. 7. Probability $P(\Lambda)$ of the observation of a conductance fluctuation Λ in the presence of single channels of the gramicidin analogue $GN^{9,15}$ before and after exposure to X-rays. The membranes were formed from monoolein/*n*-decane in the presence of 1 M CsCl. A nominally 10^{-6} M solution of $GN^{9,15}$ in 1 M CsCl (pH 3) was irradiated with 2000 Gy, a radiation dose sufficient to convert virtually all $GN^{9,15}$ to state 1 (see Figs. 3). A small amount of this solution was added to the aqueous solutions surrounding the membrane and the resulting fluctuations were observed as a function of time at a constant voltage of 50 mV. The total number of analyzed channels was 1180 (nonirradiated) and 1278 (irradiated). The mean value, $\overline{\Lambda}$, of conductance fluctuations was 44.2 pS (control) and 25.2 pS (irradiated).

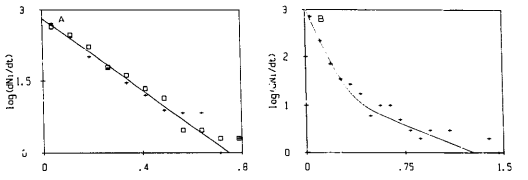

Fig. 8. Open times of $GN^{9,11,15}$-channels under the experimental conditions of Fig. 6. dN_1/dt is the number of channels per time interval, dt, showing a transition from the open (conducting) to the closed (nonconducting) state. The time $t = 0$ is defined as the transition from the closed to the open state. (A) Channels radiolyzed according to Fig. 6A as compared with the control. The total number of analyzed channels was $n = 1036$ (control) and $n = 999$ (irradiated with 2000 Gy). The mean life-time was $\tau = 115$ ms in both cases. (B) Channels photolyzed according to Fig. 6B ($n = 1132$). The data show the existence of a small subpopulation. The mean life-times are $\tau_1 = 67$ ms and $\tau_2 = 388$ ms.

Gy), was found to produce a nearly identical distribution of conductance states (Fig. 6A). Only a small shift of the maximum of the distribution to (slightly) smaller values was observed.

Channels formed by gramicidin monomers having two tryptophan residues ($GN^{9,15}$) after complete conversion to the radiolyzed state 1, showed a more pronounced shift to lower conductance values and concomitantly a broadening of the distribution (Fig. 7). This is indicative of a continuous lowering of the mean channel conductance in the radiolyzed state with an increasing number of tryptophan residues per monomer. For channels formed from normal gramicidin A the channel amplitude in state 1 could not be resolved within the experimental accuracy (see above).

Contrary to the strong influence of radiolysis and photolysis on the single-channel amplitude, there is only a relatively weak influence on the life time of the open channels. An exponential decay of the number of open channels as a function of time was observed for $GN^{9,11,15}$ in the normal (nonirradiated) state and also in the radiolyzed state 1 (Fig. 8A). The mean life-time of the channels is virtually identical for both states. Similar results were obtained with the analogue $GN^{9,15}$. The mean life-time of photolyzed $GN^{9,11,15}$-channels (Fig. 8B) is reduced by about a factor of 2. At the same time a small subpopulation of channels with a longer life time was observed.

Thus the main effect of radiolysis and photolysis found at the single-channel level is a reduction of the single-channel amplitude. The further analysis of the data will show, however, that the predominant radiation effect on the macroscopic membrane conductance is a strong reduction of the formation rate of the channels.

Discussion

Both phenomena, radiolysis and photolysis, are initiated by a modification of (at least) a single tryptophan residue of the ion channel formed by gramicidin A or by one of its analogues. The substitution of the tryptophans either by naphthylalanine, by tyrosine, by *O*-benzyl-L-tyrosine, or by phenylalanine leads to a strong reduction of the sensitivity towards both kinds of radiation (cf. Tables I and II). The nature of the molecular events finally leading to the strong decrease of the membrane conductance is, however, only partly known so far. The experiments on the gramicidin analogues with a varying number of tryptophan residues have contributed to answering some important issues. The latter may be summarized as follows:

(a) How do the different tryptophan residues of normal GA contribute to photolysis and to radiolysis? Are the two phenomena based on a cooperative action of the tryptophans?

(b) Is the decrease of the membrane conductance due to a reduced single-channel conductance, due to an increase of the rate of channel closure or due to a reduced rate of channel formation?

The answer to these questions differs for radiolysis as compared to photolysis and will be discussed in connection with the aggregational model of the gramicidin channel recently proposed [10].

(1) Radiolysis

First we will briefly summarize the results of our previous communications. Subsequently, we will show how previous ideas have to be modified and supplemented by the results of the present communication.

Our previous studies [3,4] have led to the conclusion that radiolysis of gramicidin A is due to the subsequent attack of a radical R1 (presumed to be OH or a secondary radical produced by OH, such as Cl_2^-) and a radical R2 (presumed to be HO_2) at a 'single' tryptophan residue of a gramicidin A:

$$A + R1 \rightarrow A' \quad (2)$$

$$A' + R2 \rightarrow B \quad (3)$$

The species A, A′ and B represent nonconducting gramicidin dimers (or monomers, see below), which may associate to conducting channels AA by

$$A + A \overset{K}{\rightleftharpoons} AA \tag{4}$$

Eqn. 4 has been proposed to proceed also for the species A′ and B with the same equilibrium constant K. It was assumed that the species AA, A′A and A′A′ show the same single-channel conductance, while the species AB, A′B and BB were treated as nonconducting. This assumption will have to be revised according to the result of the present communication (see below).

The model was based on the following experimental findings [3,4]:

(a) Inactivation experiments performed in the presence of different radical scavengers, in the presence and absence of oxygen, and at different pH of the aqueous solutions support the importance of OH and of HO_2 radicals for channel inactivation. Eqns. 2 and 3 also agree with a fundamental mechanism suggested for the radiolysis of the amino acid tryptophan in aqueous solution [11].

(b) The model has allowed the description of the shape of the inactivation curves at a continuous irradiation of the membrane at constant dose rate. The pronounced shoulder illustrated in Fig. 2 disappears, if (by addition of a scavenger) the concentration of one of the two types of radicals becomes rate limiting [4]. Exponential inactivation curves are obtained under these conditions, indicative of a one-hit phenomenon. If inactivation required an attack of water radicals at two (or more) loci, shoulder curves would also be observed in the presence of scavengers. Consequently, analysis of inactivation curves suggests that the attack of radicals at a single tryptophan residue is sufficient for radiolysis.

(c) The 'all-or-nothing behaviour' observed at the level of single channels agrees with the assumption of only two channel states (open and irreversibly inactivated).

(d) The model was found to provide a fair description of the time dependence of the inactivation following pulse radiolysis.

The experimental data (a–d) are in full agreement with the inactivation model depicted above. Previous data did, however, not allow a distinction between the dimer and the tetramer hypotheses of the open channel [10], i.e., Eqns. 2–4 were also found to be valid assuming the species A to represent a gramicidin monomer instead of a dimer [4].

The findings of the present paper, at least in part, seem to contradict the simple model of channel inactivation. Only partial radiolysis was observed for the analogue $GN^{9,11,15}$ (and also for $GN^{9,15}$ and $GN^{11,13}$). This indicates that the modification of more than two tryptophan residues is required for the (virtually) complete closure of the channel.

The discrepancy may be resolved, however, considering the well documented ability of biological macromolecules to allow for intramolecular energy transfer. A wealth of different studies has provided evidence (see, for example, Ref. 12) that the energy conveyed to a macromolecule by the action of ionizing radiation may be transferred over considerable distances (e.g., to the active centre of an enzyme molecule, leading to its inactivation). Starting from radical-induced changes at tryptophan residues, intramolecular radical transformations, such as electron transfer, are well established in numerous peptides [13].

According to the helical structure proposed by Urry [5], the tryptophan residues in gramicidin A are arranged in close proximity at each end of the dimer forming the channel. Since all the tryptophan residues are close to the channel mouth, all of them may be accessible to radical attack. This is supported by the largely identical inactivation behaviour of the analogues $GN^{9,15}$ and $GN^{11,13}$, with two tryptophan residues in positions 9 and 15 and in positions 11 and 13, respectively. The close sequential and spatial arrangement of the (maximum) four tryptophan residues favours energy transfer between these groups.

Therefore, supplementing the simple model of channel radiolysis (and at the same time answering question (a)) in the first part of this section), we suggest that a radical-induced modification of one of the tryptophan residues leads, by way of intracellular energy transfer, to a modification of all tryptophan residues of the monomer. We think that radical attack according to Eqns. 2 and 3 initiate a series of radical transformations

$$B \rightarrow \cdots \rightarrow R \tag{5}$$

which, via an unknown number of intermediate products, finally leads to the radiolyzed state R of gramicidin. The nature of R depends on the number N of tryptophan residues per gramicidin monomer.

We now turn to question (a) and we try to analyze state 1, i.e., the state of radiation independent membrane conductance observed at very large radiation dose (See Figs. 3 and 4). For $N = 3$ or $N = 4$ (i.e. for normal gramicidin A), I_1 is determined by the conductance of the unmodified (pure) lipid bilayer. For $N \leq 2$, however, I_1 is clearly different from the basic conductance of the pure lipid bilayer, i.e., I_1 is determined by channels formed by radiolyzed gramicidin. Therefore, contrary to normal GA, the nature of the radiolyzed channels of the analogues $GN^{9,11,15}$, $GN^{9,15}$ and $GN^{11,13}$ can be studied.

The membrane conductance, λ, is determined by the product of the single-channel conductance, Λ, and of the number of channels, N_o, in the open state:

$$\lambda = \Lambda N_o \tag{6}$$

For $GN^{9,11,15}$ ($GN^{9,15}$) the ratio $\overline{\lambda}(0)/\overline{\lambda}(1)$ of the mean values of the amplitudes of conductance fluctuations between the normal state 0 and state 1 is only 1.21 (1.75) (see Figs. 6A and 7). Similar values were obtained, if the analogues were incorporated in membranes formed from diphytanoylphosphatidylcholine.

The ratio $\lambda(0)/\lambda(1)$ of the macroscopic membrane conductances in the presence of $GN^{9,11,15}$, on the other hand, corresponds to about 2–20 depending on the lipid environment of the channel (see Figs. 3 and 4). In the case of the analogue $GN^{9,15}$ the ratio is even ≥ 100. Consequently, applying Eqn. 6, the main effect of ionizing radiation on the transport system under study is a reduction of the number N_o of open channels.

The gramicidin concentration inside the membrane may be considered as constant throughout an inactivation experiment. This is concluded from the time-independent conductance observed at large radiation doses. Therefore, the number N_o of open channels is determined by the rates of channel formation and channel dissociation only. This holds irrespective of the structure and stoichiometry of the channel and of the molecular nature of the opening and closing events. The transition rate from the open into the closed state (i.e., the inverse of the mean life-time in the open state) is found to be virtually identical in state 1 as compared to the control (see Fig. 8A).

Consequently, a reduction of the formation rate of open channels must be responsible for the decrease of N_o, i.e., for the main part of the radiation-induced decay of the current.

A reduced rate of channel formation of irradiated gramicidin also allows an explanation of the dependence of the radiation-sensitive fraction, Θ, on the lipid environment of the channels (cf. Figs. 3 and 4): The formation of open channels is closely related to the formation of aggregates (compare Eqn. 4 and the discussion below).

Aggregate formation could be influenced by structural parameters of the surrounding lipid matrix. The effect of the latter might be different for irradiated gramicidin as compared with the control.

The experimental finding of a decreasing number, N_o, of open channels with increasing radiation dose is in contrast to our previous assumption of an identical equilibrium constant K of gramicidin aggregation for the species A and B (cf. Eqn. 4). It was found, however, that this assumption is of minor importance for the mathematical description of our previous inactivation data (see Appendix).

(2) Photolysis

Contrary to radiolysis, photolysis was found to be roughly proportional to the number of tryptophan residues (see Table II). Moreover, the inactivation process proceeds via one or several states of reduced channel conductance as was detected by single-channel experiments [1]. The present study has shown that the shift to lower conductance values of partially photolyzed gramicidin and the concomitant broadening of the distribution of single-channel amplitudes is observed also for $GN^{9,11,15}$, i.e., in the presence of only one tryptophan residue per monomer (Fig. 6B). Though radiolyzed $GN^{9,15}$ shows a similar behaviour (Fig. 7), the two phenomena differ at least in one important aspect: While Fig. 7 shows the single-channel distribution after complete conversion to the radiolyzed state 1 observed at a 'saturating' radiation dose, Fig. 6B illustrates a transient state of partially photolyzed $GN^{9,11,15}$, which after prolonged exposition to UV light leads to complete channel closure. Thus photolysis is a multi-photon process. This holds even in the presence of only one tryptophan residue per monomer, since a qualitatively similar behaviour was found for $GN^{9,11,15}$ and GA. Further differences between radiolysis and photolysis are as follows (data not presented in detail):

(a) Contrary to radiolysis, which shows a pronounced pH-dependent [3], the effect of UV light does not depend on the pH value of the aqueous phase (pH 3–pH 10).

(b) While the effect of ionizing radiation is virtually negligible in the absence of oxygen, the sensitivity towards UV light rather is increased, if the oxygen concentration is reduced to the one percent level.

These findings indicate a different inactivation mechanism for photolysis. Though both phenomena, radiolysis and photolysis, start at the same amino acid residues of the peptide, the process of photolysis seems to proceed according to a different reaction path as compared with radiolysis. As a final result of completely photolyzed gramicidin, the amplitude of the single-channel conductance is virtually zero even at a single tryptophan residue per monomer. A similar estimate as in the case of radiolysis shows, however, that the decay of the membrane conductance, apart from a reduced single-channel conductance, is also strongly influenced by a reduced rate of channel opening.

The mathematical form of the time dependence of photolytic inactivation described by Eqn. 1 does not depend on the number of tryptophans per monomer. It may be interesting to note in this context that the decay of the tryptophan fluorescence accompanying photolysis of di- or tripeptides in aqueous solution was also found to obey Eqn. 1 [14]. In the case of normal gramicidin A a close correlation between the rates of fluorescence loss and the rate of channel inactivation was observed [2]. Thus, the two exponential terms mirror a property of the single tryptophan residue. The presence of more than one tryptophan residue (within the experimental accuracy) only leads to a change of the characteristic time constants (see Table II).

The differences observed in the mechanisms of radiolytic and photolytic channel inactivation mirror the different nature of the inactivation process. While photolysis is a direct radiation effect initiated by light absorption of the target group, radiolysis of gramicidin channels is based on the radiation chemistry induced by water radicals. The individual molecular steps of the inactivation processes, including the detailed nature of the chemical products finally produced, are so far unknown.

(3) Consequences for the channel structure

The single-channel fluctuations observed in the presence of gramicidin A are frequently explained on the basis of the statistical fluctuations accompanying a monomer-dimer equilibrium:

$$M + M \rightleftharpoons D \qquad (7)$$

where D is believed to represent the open channel. Indeed, evidence from different experimental approaches, such as the concentration dependence of the conductance [15–17], voltage-jump relaxation experiments [18], noise analysis [19,20], and the observation of hybrid channels [16,21,22] has supported the idea that a dimerization reaction represents an essential step of the formation of the open channel.

It has been found, however, that the process of channel activation is more complicated than assumed so far [10]. Based on temperature-jump and voltage-jump experiments, the kinetics of channel formation was shown to represent a multistate phenomenon governed by at least two different relaxation times. Since the relaxation behaviour was found to be largely identical for normal gramicidin A and for chemically dimerized malonyl-bis(desformylgramicidin), an aggregate of two parallel dimers was suggested to represent the smallest unit of the open channel. The dimerization process mentioned above (Eqn. 7) was interpreted as an association of two nonconducting dimers D to a tetramer T (compare Eqn. 4). Aggregate formation was suggested to be accompanied by the opening of the aqueous channel inside one or both of the dimers.

The possibility of aggregate formation by dimer-dimer interactions was first discussed by Urry [6]. Based on conformational studies, aggregate formation (up to the formation of a supramolecular structure of parallel rows of aggregates of gramicidin dimers) was proposed to be due to tryptophan–tryptophan interactions of neighbouring dimers. Experimentally, aggregation was observed by different methods: Fluorescence quenching due to Trp-Trp interactions [23], dielectric relaxation studies of lysophosphatidylcholine-packaged gramicidin channels [24], and electron microscopic studies of gramicidin A incorporated into dispersions of lysophosphatidylcholine [25]. It was found that the tendency of gramicidin to self-associate may even strongly influence the structure of lipid membranes, changing the temperature of phase transitions or triggering the transition of a micellar structure into that of a bilayer structure (for a review see Ref. 26).

The effects mentioned above were observed at concentrations far above those usually applied throughout conductance measurements. The process of formation of a supramolecular structure will, however, extend over many orders of magnitude in the concentration, resembling the formation of micelles in aqueous solutions. The association of dimers to tetramers may be considered as the first step of a complex aggregation process of the dimers occurring at extremely small concentrations and resembling the formation of nuclei, the growth of which is induced by an increase of the concentration.

The aggregational model of channel formation obtains further support by the radiolysis experiments of the present study. The inactivation of channels formed by the analogues $GN^{9,15}$ and $GN^{9,11,15}$ is, as shown above, mainly due to a reduced opening rate of the channels. This finding is difficult to explain in the frame of the monomer-dimer hypothesis. The radical-induced modification of a Trp-residue occurs at the C-terminal end, near the membrane/water interface. For the association of monomers to dimers, however, the N-terminal end located in the membrane interior is responsible. The aggregational model of channel opening, on the other hand, is based on dimer-dimer interactions for which Trp-Trp contacts have been suggested to be of great relevance (see above). Thus, the radiation induced reduction of the rate of channel formation (i.e. the decrease of the aggregation rate) becomes plausible.

There is a further argument supporting the idea that aggregation of dimers is important for the channel opening. It is based on a comparison of the UV-sensitivity of gramicidin channels and of sodium channels of excitable membranes. Studies by different authors [27–31] have shown, that sodium channels of different organisms may be photochemically inactivated. Conti et al. [31] argued that photolysis of a single tryptophan residue might be responsible for the effect. The UV-sensitivity of gramicidin A [1] is about 18-times larger than that of sodium channels from myelinated nerve [29] and about 23-times larger than the UV-sensitivity of sodium channels from squid axon [31]. The numbers refer to the inactivation constant γ at a wavelength of 280 nm. γ is defined through Eqn. 8:

$$I(t)/I(0) = \exp(-\gamma i_r t) \qquad (8)$$

$I(t)$, membrane current at time t; i_r, radiant intensity:

(GA) = 0.028 $cm^2 \cdot mW^{-1} \cdot s^{-1}$ [1], (SC) = 1.52 $\cdot 10^{-3}$ $cm^2 \cdot mW^{-1} \cdot s^{-1}$ [29],
(SC) = 1.22 $\cdot 10^{-3}$ $cm^2 \cdot mW^{-1} \cdot s^{-1}$ [31] (SC, sodium channel).

In the case of gramicidin A Eqn. 8 represents an approximation (see Eqn. 1).

Two effects may contribute to explain the different UV-sensitivity of sodium and gramicidin channels: A different number of tryptophan residues per channel and a different quantum efficiency of the photochemical effect. If the latter is identical for both types of channels, a gramicidin aggregate forming an open channel has to consist of two or three dimers at least to account for the different sensitivity.

Though this argument, repeatedly used in the literature, is in qualitative agreement with the aggregational model, it does not, however, represent a definite determination of the size of the aggregate. This would require a measurement of the quantum efficiency, as was performed at the photolysis of the amino acid tryptophan and analogous compounds [32,33].

Appendix

The influence of gramicidin aggregation on the mathematical form of inactivation curves

The time dependences, $I(t)$, of the electric current following pulse radiolysis or continuous radiolysis may be expressed by

$$I(t)/I_0 = \left[\exp(-\beta D) + (1-\exp(-\beta D))\left(\frac{\gamma/D}{\gamma/D+1-\exp(-\tilde{k}t)}\right)\right]^{\Omega} \qquad \text{(A-1)}$$

and

$$I(t)/I_0 = \left[\frac{a_2}{a_2-a_1}\exp(-a_1 t) - \frac{a_1}{a_2-a_1}\exp(-a_2 t)\right]^{\Omega} \qquad \text{(A-2)}$$

For a derivation of Eqns. A-1 and A-2, including the definition of the symbols, see Ref. 4. Both equations were obtained under the conditions outlined in Discussion, i.e., assuming the same association behaviour for the species A, A' and B (cf. Eqn. 4). $\Omega = 2$ is found in this case.

The experiments with the analogue GN[9,11,15] have shown, however, that the association tendency of the species B is considerably smaller compared to that of the species A. In the following we neglect the association of B. By using the same procedure as in Ref. 4, one finds that for two limiting cases Eqns. A-1 and A-2 are still valid, however, with a different value for the parameter Ω in one of the two cases. If the number, N_A, of dimers A is considerably larger than the number, N_{AA}, of tetramers AA (i.e., $N_A \gg N_{AA}$), $\Omega = 2$ is again found. For $N_{AA} \gg N_A$, on the other hand, $\Omega = 1$ is obtained. The experimental data presented in Ref. 4 may be described setting $\Omega = 2$ or $\Omega = 1$, with only minor changes of the other parameters required. Therefore, the data do not allow the prediction of the association behaviour of the species B.

Acknowledgements

We are obliged to Dr. A. Henglein for giving us the opportunity to use the facilities of the Hahn-Meitner-Institut. We thank Dr. Y. Trudelle, Centre de Biophysique Moléculaire, CNRS, Orléans for providing us with samples of the gramicidin analogues GT and GT(Bzl). The performance of the single-channel measurements has greatly profited from the expert advice of Dr. H.-J. Apell, Universität Konstanz. We thank Dr. R. Clarke for carefully reading the manuscript. The study was supported by the Ministerium für Wissenschaft und Kunst Baden-Württemberg and by the Deutsche Forschungsgemeinschaft (Az. Sta 236/2).

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