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Radiation inactivation of ion channels formed by gramicidin A. Protection by lipid double bonds and by α-tocopherol

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The conductance induced by the channel-forming peptide gramicidin A in lipid membranes is reduced by many orders of magnitude on exposure of the membrane and its aqueous environment to ionizing radiation. This results from an interaction of free radicals of water radiolysis with the tryptophan residues of gramicidin A. The sensitivity of the ion channels towards irradiation is strongly reduced in the presence of either vitamin E or of highly unsaturated lipids. An increase of the D_{37} dose up to a factor of 50 was found. The phenomena are interpreted via a reduction of the effective concentration of free radicals (such as $OH^{\cdot}$) in the membrane by reaction with unsaturated fatty acid residues or with vitamin E.

Introduction

The pentadecapeptide gramicidin A is known to form ion channels for monovalent cations in biological membranes and in artificial lipid membranes. Due to the numerous investigations of its structure and transport properties (for a review, see Refs. 1–9), the gramicidin channel is usually considered as a well-characterized model of the more complicated ion channels of biological membranes.

We have been studying the effect of ionizing radiation on this model channel in order to learn something about the principles of how ion transport across biological membranes may be affected by irradiation [10–12]. We have found that the gramicidin channels may be inactivated by comparatively small doses of radiation under appropriate experimental conditions. The inactivation depends on the simultaneous presence of $OH^{\cdot}$ and of $HO_2^{\cdot}$-radicals (generated by water radiolysis) and is strongly coupled to the presence of the tryptophan residues of gramicidin A.

The present communication is concerned with the role of certain membrane constituents which serve as protective agents for the gramicidin channel. The well-known radical scavenger α-tocopherol (vitamin E) as well as unsaturated fatty acid side chains of the membrane lipids are shown to function as effective inhibitors of the radiation effect.

Materials and Methods

Optically black planar lipid membranes were formed from a 1% solution of lipid in *n*-decane (Fluka, Buchs, Switzerland; standard for gas chromatography). The lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Those having more than one double bond per fatty acid residue (dilinoleoylphosphatidylcholine, dilinolenoylphosphatidylcholine and diarachidonoylphosphatidylcholine) were kept under argon and were routinely tested for oxidation by spectrophotometric detection of conjugated dienes at 233 nm [13,14] and by measurement of malondialdehyde formation (dilinolenoylphosphatidylcholine and diarachidonoylphosphatidylcholine) [15]. α-Tocopherol was purchased from Fluka (Buchs, Switzerland).

A PTFE-cuvette for the formation of horizontal lipid membranes was used, which allowed the maintenance of a thin water layer above the membrane (1–3 mm) providing a very small attenuation of the X-ray intensity (c.f. Fig. 1). The cuvette was arranged within a lead shield and was irradiated with 80 kV X-rays filtered by 0.3 mm Al (Philips-Müller RT100).

Inactivation of gramicidin channels was studied by application of a constant voltage of 50 mV and measurement of the membrane current as a function of time. The current, I (after amplification) and the dose

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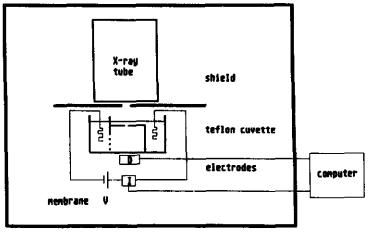

Fig. 1. Schematic illustration (not to scale) of the experimental set-up applied to study the effect of ionizing radiation on the conductance of planar (black) lipid membranes. The current, I, at a constant voltage, V, and the dose rate, dD/dt, (via ionization chamber D) are monitored as a function of time. The data are transferred into a computer for further analysis.

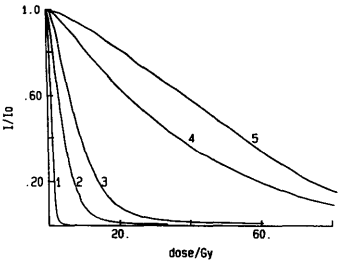

Fig. 2. Radiation inactivation of ion channels formed by gramicidin A in phosphatidylcholine membranes with a different degree of unsaturation: (1) diphytanoylphosphatidylcholine, (2) dioleoylphosphatidylcholine, (3) dilinoleoylphosphatidylcholine, (4) 4:1-mixture of dilinolenoylphosphatidylcholine and diphytanoylphosphatidylcholine, (5) 7:3-mixture of diarachidonoylphosphatidylcholine and diphytanoylphosphatidylcholine. I_0 is the current before irradiation.

signal – obtained from an ionization chamber (PTW DL 4, Physikalisch-Technische Werkstätten Dr. Pychlau, Freiburg, F.R.G.) – were fed into a computer (Compaq 386/20) equipped with analog/digital board DAS16F (MetraByte). The data were analyzed by using the software package Asyst.

The aqueous solution in which the membranes were formed contained 1M NaCl (Merck, Suprapur) at pH 3 (unbuffered, air-saturated). The pH and the oxygen concentration are important parameters, which determine the radiation sensitivity of gramicidin A [10,11]. Commercially available gramicidin (Sigma), a mixture of gramicidins A, B and C at approximate ratios 8:1:2 was added to the membrane forming solution (10^{-6}–10^{-7} M). The presence of the organic solvents methanol and chloroform was carefully avoided, since both substances represent well-known radical scavengers. The membrane conductance before irradiation was in the range 10^{-4}–10^{-3} Ω^{-1} cm^{-2}. The temperature was 20°C.

The applied dose rate, $\dot{D}$, (varied between 1–20 Gy/min) was chosen in such a way that the D_{37} dose was achieved within several minutes.

Results

The membrane conductance induced in the presence of the pore-forming substance gramicidin A will be (virtually) decreased to the basic conductance of the pure lipid bilayer, if the membrane and its aqueous environment is exposed to ionizing radiation [10–12]. The sensitivity of the channels is, however, strongly dependent on the composition of the lipid bilayer (c.f. Fig. 2 and Table I). The radiation dose, D_{37}, necessary to reduce the membrane conductance to 37% of its original value, depends on the number and on the position of the double bonds of the fatty acid residues of the lipids. The D_{37} dose will be larger by almost two orders of magnitude, if the membrane is formed from solutions, which contain a large percentage of highly unsaturated lipid (dilinolenoyl- or diarachidonoylphosphatidylcholine) as compared with membranes formed exclusively from saturated branched chain diphytanoylphosphatidylcholine. This indicates that the ion channels are protected against the radiation effect in the presence of lipid double bonds.

The relationship between the D_{37} dose and the mole fraction, x_i, of unsaturated lipid shows a complicated behaviour (see Fig. 3). At low and at high mole fractions, x_i, the data agree with a linear relationship.

TABLE I

The radiation dose D_{37} for inactivation of ion channels formed by gramicidin A in membranes of different phosphatidylcholines

The D_{37} dose is defined as the radiation dose necessary to reduce the membrane conductance to 37% of its original value. The data represent mean values of typically five membranes (±S.D.). The lipids are characterized by their fatty acid residues and (apart from the saturated branched chain phytanic acid) by the number and positions of their double bonds (in brackets).

Fatty acid residues	D_{37}(Gy)
Diphytanoyl (3,7,11,15-C(16:4CH_3)	1.38±0.28
Dipetroselinoyl (Δ^6-$C_{18:1}$)	4.1 ±0.6
Dioleoyl (Δ^9-$C_{18:1}$)	5.5 ±0.6
Dielaidoyl (*trans* Δ^9-$C_{18:1}$)	5.5 ±0.9
Dilinoleoyl ($\Delta^{9,12}$-$C_{18:2}$)	10.3 ±2.3
Dilinolenoyl ($\Delta^{9,12,15}$-$C_{18:3}$)	49 [a]
Diarachidonoyl ($\Delta^{5,8,11,14}$-$C_{20:4}$)	78 [a]

[a] Values extrapolated from Fig. 3 ($x(i) = 1$).

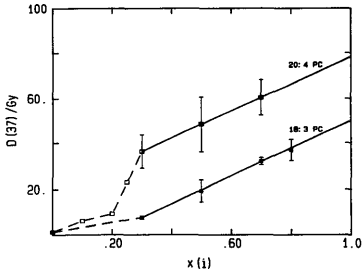

Fig. 3. The sensitivity of gramicidin channels (expressed via the D_{37} dose) as a function of the mole fraction $x(i) = n(i)/(n(i) + n(\text{dpl}))$ of an unsaturated lipid, i, in a saturated lipid (dpl) (dpl = diphytanoylphosphatidylcholine, i = dilinolenoylphosphatidylcholine (18:3-PC) or diarachidonoylphosphatidylcholine (20:4-PC)). The bars indicate the standard deviation observed with at least five different membranes. The solid lines represent least square fits to the data assuming a linear relationship between D_{37} and x_i. The experimental conditions were as described in the legend to Fig. 1.

There is a transition between the two regions at $x_i = 0.2–0.3$, which may indicate a lateral phase separation inside the membrane (see below). Membranes formed exclusively from dilinolenoyl- or diarachidonoylphosphatidylcholine were found to be rather unstable. Therefore, the corresponding D_{37} values of Table I were obtained by extrapolation from Fig. 3 ($x(i) = 1$).

Gramicidin channels also appear to be protected against the effect of ionizing radiation in the presence of α-tocopherol (c.f. Fig. 4). As in the case of unsaturated lipids, channel inactivation will be observed at higher radiation doses, if the concentration of α-tocopherol is increased. There is, however, an additional phenomenon in this case, namely the appearance of a more pronounced shoulder in the inactivation curves. There is a threshold of the radiation dose, below which the channels appear virtually insensitive towards an irradiation. The threshold increases with the concentration of vitamin E. No threshold is observed in the absence of vitamin E. The inactivation curves measured in the presence or absence of polyunsaturated lipids show a continuous decrease even at low radiation doses (not shown in Fig. 2).

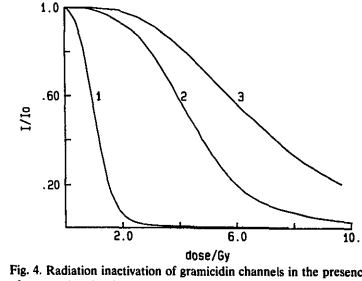

Fig. 4. Radiation inactivation of gramicidin channels in the presence of α-tocopherol. The membranes were formed from solutions of diphytanoylphosphatidylcholine (1%) and α-tocopherol in n-decane (molar ratio 1:0 (1), 5:1 (2) and 3:1 (3)). The D_{37} dose values obtained are 1.2 Gy (1), 5 Gy (2), and 7.6 Gy (3).

Discussion

Gramicidin A is the first ion channel whose mechanism of inactivation by ionizing radiation has been studied in some detail [10–12]. The radiation-induced decay of the membrane conductance has been shown to be due to a decrease of the single channel conductance and also due to a reduction of the formation rate of open ion channels.

Inactivation proceeds by an indirect radiation effect, i.e. by a reaction of free radicals of water radiolysis with one of the tryptophan residues of gramicidin A. The process of inactivation starts by attack of $OH^{\cdot}$ radicals (or of secondary radicals produced by $OH^{\cdot}$, such as $Cl_2^{\cdot-}$) which is followed by a reaction with $HO_2^{\cdot}$ radicals:

$$A + R_1 \rightarrow A' \quad (1)$$

$$A' + R_2 \rightarrow B \quad (2)$$

A = gramicidin A, $R_1 = OH^{\cdot}$ ($Cl_2^{\cdot-}$), $R_2 = HO_2^{\cdot}$, B = inactivated state.

Eqns. 1 and 2 have been tested by the action of radical scavengers, by the analysis of the shape of inactivation curves as well as by studying the kinetics of inactivation by using the method of pulse radiolysis [11]. The experiments agree with the assumption that reactions (1) and (2) with a single tryptophan residue of a gramicidin channel are sufficient to convert the channel into a state of virtually zero conductance. This holds for normal gramicidin A with four tryptophan residues per monomer. In the case of gramicidin analogues having only one or two tryptophan residues per monomer, inactivation leads to a reduced single-channel conductance. The necessity of more than one tryptophan residue per monomer for complete channel inactivation – in combination with a single residue that has to be modified by radical attack only – has been explained by intramolecular energy transfer leading to a chemical modification of all tryptophan residues of the respective monomer [12].

The free radical $OH^{\cdot}$ – apart from its importance for the inactivation of transport systems inside a lipid bilayer – is well-known for its ability to initiate lipid

peroxidation (for a review, see for example, Refs. 16–21). This process, by way of a radical chain mechanism, leads to the formation of lipid peroxides, and finally to short chain decomposition products such as aldehydes and alkanes. A simplified model of the radical chain mechanism explaining the formation of lipid peroxides and including initiation, propagation and termination is given by Eqns. 3 to 6:

Initiation (by a radical species $X^{\cdot}$, e.g. $OH^{\cdot}$):

$$LH + X^{\cdot} \xrightarrow{k_i} L^{\cdot} + XH \quad (3)$$

Propagation of the chain:

$$L^{\cdot} + O_2 \xrightarrow{k_{ii}} LOO^{\cdot} \quad (4)$$

$$LOO^{\cdot} + LH \xrightarrow{k_p} LOOH + L^{\cdot} \quad (5)$$

Termination of the chain:

$$LOO^{\cdot} + LOO^{\cdot} \xrightarrow{k_t} \text{nonradical product} \quad (6)$$

It has been found that radiation-induced lipid peroxidation has a profound influence on ion transport across lipid membranes mediated by macrocyclic ion carriers [22].

Lipid peroxidation is most efficient with lipids having polyunsaturated fatty acid residues, i.e. this phenomenon is strongly augmented in the presence of double bonds. As a consequence the concentration of the initiating species, $OH^{\cdot}$, inside the membrane may be expected to be lowered by increasing the number of double bonds per lipid molecule. Therefore, the chemical modification, by attack of $OH^{\cdot}$, of other membrane constituents will be diminished. The protection of gramicidin channels against ionizing radiation, observed in the presence of unsaturated fatty acid residues (c.f. Figs. 2 and 3 and Table I) is thought to represent an example of a possibly general mechanism.

The spatial arrangement of the channel including the location of its tryptophan residues is illustrated in Fig. 5. It is generally believed that the gramicidin channel is represented by a dimer formed by head to head association of two monomers [23]. The dimeric structure shows the phenomenon of self-association [24,25] which seems to be related to the process of channel opening [12,26] (not shown in Fig. 5). The length of the dimer of about 25–30 Å is considerably shorter than the thickness of a lipid bilayer formed from *n*-decane [27]. The four tryptophan residues of each monomer are located at the channel mouth. On the one hand this position allows free access of radicals from the aqueous phase. On the other hand it is in close contact with the region of double bonds of the lipid bilayer which is responsible for the protective

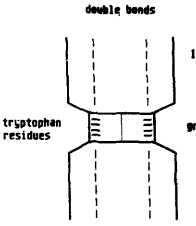

Fig. 5. Schematic arrangement of a gramicidin dimer in a lipid bilayer membrane with one double bond per fatty acid residue.

effect discussed above. Protection (expressed via the D_{37} dose, c.f. Table I) increases with the number of double bonds per fatty acid residue. The D_{37} dose is identical for oleoyl- and elaidoyl-residues (i.e. *cis*- versus *trans*-residues) and is slightly smaller for dipetroselinoylphosphatidylcholine as compared with dioleoylphosphatidylcholine.

Protection against free radical attack will show a discontinuity, if membranes are formed from mixtures of diphytanoylphosphatidylcholine and diarachidonoylphosphatidylcholine (c.f. Fig. 3). Two alternative interpretations may be envisaged to explain this phenomenon:

(1) The increase of the D_{37} dose at $x_i = 0.3$ may be understood by assuming a selective enrichment of diarachidonoylphosphatidylcholine around the gramicidin channels for $x_i \geq 0.3$. The formation of an annulus of unsaturated fatty acid residues around the channel certainly would increase the efficiency of protection.

(2) The mixture of the two lipids shows the phenomenon of a lateral phase separation leading to the formation of two separate phases at large x_i. The two phases differ with respect to their content of diarachidonoylphosphatidylcholine. Gramicidin channels are preferentially formed in the phase enriched in the unsaturated lipid.

Both alternatives would give rise to an increase of the number of lipid double bonds in the neighbourhood of the channels and thus would lower the effective concentration of $OH^{\cdot}$ radicals at the tryptophan residues of the channel.

Protection against free radical attack is also observed in the presence of vitamin E (α-tocopherol) (Fig. 4). The latter is a well-known lipid soluble antioxidant (for a review, see Refs. 28 and 29). Vitamin E has been found to react with both types of radicals, $OH^{\cdot}$ and $HO_2^{\cdot}$ [30], which – according to Eqns. 1 and 2 – are responsible for gramicidin inactivation:

$$OH^{\cdot} + ATOH \rightarrow ATO^{\cdot} + H_2O \quad (7)$$

$$HO_2^{\cdot} + ATOH \rightarrow ATO^{\cdot} + H_2O_2 \quad (8)$$

ATOH = α-tocopherol.

It has been found that vitamin E also reacts with lipid peroxyl radicals. The resulting vitamin E radical, $ATO^{\cdot}$, contrary to lipid peroxyl radicals, $LOO^{\cdot}$, is not able to abstract hydrogen atoms from fatty acid side chains. Therefore, vitamin E not only reduces the rate of initiation but also interrupts chain propagation during lipid peroxidation (c.f. Eqns. 3–5).

Thus protection of gramicidin channels by vitamin E against the effect of ionizing radiation is caused by the same effect as protection by unsaturated lipids, namely by a reduction of the effective concentration of free radicals at the entrance of the channel.

There is a greater efficiency of α-tocopherol as compared with unsaturated lipids at small doses of radiation. This is concluded from the pronounced shoulder, observed in the presence of vitamin E (c.f. Fig. 4). There is hardly a radiation effect up to about 2 Gy at a molar ratio lipid/vitamin E (in the membrane forming solution) of 3 : 1. Similar results were obtained throughout the study of the inhibitory effect of vitamin E on lipid peroxidation [28,31]. Two different factors seem to contribute to the extraordinary inhibitory effect of vitamin E, namely high reaction rates of Eqns. 7 and 8, and the comparatively large lateral diffusion coefficient of vitamin E [32], which exceeds that of phospholipid molecules by one to two orders of magnitude.

The relative potency of the inhibitory effects of vitamin E and of polyunsaturated lipids is, however, changed at comparatively large radiation doses. The D_{37} doses observed in the presence of diarachidonoylphosphatidylcholine are substantially larger compared with those found in the presence of vitamin E. This indicates a smaller 'inhibitory capacity', of vitamin E, being exhausted at larger radiation doses.

Polyunsaturated fatty acid residues (PUFAs) of membrane lipids are usually considered as components amplifying the deleterious effects of ionizing radiation on biological membranes. This is largely based on radiation induced lipid peroxidation which is strongly enhanced in the presence of PUFAs. The present study indicates, however, that PUFAs may reduce the radiation effect on other membrane components. In this way specific membrane functions could be protected at the cost of a damage of the lipid matrix of biological membranes.

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