Inactivation by ionizing radiation of ion channels formed by polyene antibiotics amphotericin B and nystatin in lipid membranes: An inverse dose-rate behavior

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ABSTRACT The phenomena reported are part of a study about the effects of ionizing radiation on membrane transport. We found that the conductance of lipid membranes in the presence of the polyene-antibiotics nystatin or amphotericin B is reduced to virtually zero following irradiation. Ion channels formed by these substances seem to represent extremely sensitive structures being inactivated by radiation doses in the range of a few Centigray (1 cGy = 1 rad) at sufficiently small dose rates. Inactivation shows a so-called inverse dose-rate behavior, i.e., at constant radiation dose the effect increases with decreasing dose rate. Similar to radiation-induced lipid peroxidation the phenomenon may be understood on the basis of a radical chain mechanism initiated by free radicals of water radiolysis. The process—via peroxidation of the polyene part of the molecules—is suggested to modify the hydrophobic exterior and to destabilize the barrel-like structure of the ion channels.

INTRODUCTION

Biological membranes have repeatedly been considered as an important target for the development of cellular radiation damage (1–4). The molecular basis of radiation action on membrane transport, however, has remained rather obscure. We have been studying the effect of ionizing radiation on a series of model compounds, which have been well-characterized with respect to their ability to increase the ion permeability of biomembranes and of artificial lipid membranes. The radiation-induced modification of ion transport, induced by channel-forming substances or by ion carriers in lipid membranes, may serve as a clue for possible radiation effects on more complicated transport structures of biological membranes.

The conductance of planar (black) lipid membranes in the presence of model compounds was observed to increase or to decrease by several orders of magnitude on irradiation of the membrane and its aqueous environment (for a review see (5)). The function of a transport system may be influenced by ionizing radiation in two ways: *a*) The efficiency of a system may increase or decrease due to a modification of the lipid matrix (by radiation induced lipid peroxidation). This was found to be the case in the presence of ion carriers of the valinomycin type (6). *b*) The system may become inactivated. Inactivation may result from a chemical reaction with free radicals generated by water radiolysis. The membrane conductance induced by gramicidin A is reduced by many orders of magnitude by a reaction of $OH^{\bullet}$ and of $HO_2^{\bullet}$ radicals with the tryptophan residues of the peptide (7–9). The hydroxyl radical $OH^{\bullet}$ and the perhydroxyl radical $HO_2^{\bullet}$ (as well as other oxygen radicals) are well known for their deleterious cellular effects and have been discussed in the context with a variety of diseases (10).

The present contribution reports about an extreme sensitivity of the conductance induced by amphotericin B or nystatin in planar lipid membranes, if the latter are irradiated by x-rays or by fast electrons from a linear accelerator. Both substances belong to the same class of polyene antibiotics. They are characterized by lactone rings, which contain a series of double bonds (a heptaene in the case of amphotericin B and a tetraene and a diene in the case of nystatin). It is these special structural characteristics, which account for the high reactivity of the compounds towards radiation induced free radicals and are finally responsible for the strong changes of the ion permeability observed at irradiation of polyene doped lipid bilayers.

MATERIALS AND METHODS

Planar lipid membranes were formed from a mixture of lipid and cholesterol dissolved in *n*-decane (standard for gas chromatography; Fluka, Buchs, Switzerland) in aqueous solutions containing appropriate amounts of either amphotericin B or nystatin. The lipids (dioleoyllecithin, diphytanoyllecithin, dilinoleoyllecithin, and dilinolenoyllecithin) were obtained from Avanti Polar Lipids (Birmingham, AL, USA), the polyene antibiotics from Sigma Chemie (Deisenhofen, Germany). Stock solutions of the latter in dimethylsulfoxide (DMSO) were prepared every 1–2 wk and were kept light-protected at −20°C. Lipid membranes were formed in aqueous solutions (prepared daily) of either 0.1 M or 1 M NaCl (usually pH 3, unbuffered) to which small amounts of the stock solutions of the antibiotics were added. Their concentration was chosen to obtain a membrane conductance in the range of 10^{-4}–$10^{-2}\ \Omega^{-1}\ cm^{-2}$ (about 50–500 ng/ml amphotericin B or 1–3 μg/ml nystatin, depending on the concentration of cholesterol and on the salt concentration). The maximum concentration of DMSO in water was less than 30 mM (less than 100 μl DMSO per 50 ml water).

Irradiation of the membranes and their aqueous environment by 80 kV x-rays was performed as described in detail in a previous publication (11). A PTFE-cuvette was used for the formation of horizontal lipid membranes (of 1 mm diameter). Thereby, the water layer above the membrane could be kept sufficiently thin (1–3 mm) to ensure a

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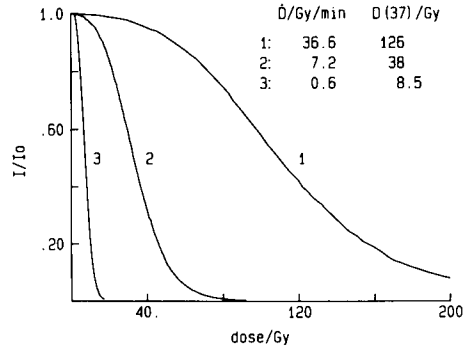

FIGURE 1 Inactivation by 80 kV x-rays of ion channels formed by the antibiotic amphotericin B in planar lipid membranes. The radiation-induced decay of the electric current, I, at a constant voltage of 50 mV, was followed as a function of the radiation dose. The original conductance (I_0 = current before irradiation) was between 10^{-3}–10^{-2} Ω^{-1} cm^{-2}. Experiments were performed at different dose rates, $\dot{D}$. Membranes were formed from solutions of 1% lecithin and 0.8% cholesterol in n-decane. The aqueous solutions separated by the membrane contained 0.1 M NaCl (pH 3, unbuffered, air-saturated) and 50 ng/ml amphotericin B.

small attenuation of the x-ray intensity. The dose rate—measured with an ionization chamber—was varied via the current amplitude of the x-ray tube (Philips-Müller RT100; Philips, Freiburg, Germany), by changing the distance to the cuvette and the thickness of an additional Cu-Filter (0–3 mm). The maximum dose rate was about 40 Gy/min.

Larger dose rates were obtained by using sequences of pulses of 14 MeV electrons (pulse length 10 ns, repetition time 40 ms) supplied by the linear accelerator (LINAC) of the Hahn-Meitner-Institut. The dose rate was varied via the pulse amplitude in this case. Average dose rates (including the repetition time) of up to 10^4 Gy/min were used. For further details of the method of pulse radiolysis (as applied at planar lipid membranes) the reader should consult references (8) and (12).

Independent of the method of irradiation, the membrane conductance was monitored as a function of time via the electric current at a constant voltage of 50 mV. The data (current and dose rate) were fed into a computer and were analyzed by using the software package Asyst (Keithley Instruments, Germering, Germany). By integrating the dose rate the conductance was obtained as a function of the radiation dose.

Membranes were normally formed in air-saturated aqueous solutions. In some experiments the oxygen concentration was reduced by one to two orders of magnitude by flushing the cuvette with argon (12). All experiments were performed at 20°C.

RESULTS

If lipid membranes, doped with channels from either amphotericin B or nystatin, are irradiated by 80 kV x-rays, a strong decrease of the membrane conductance is observed (see Fig. 1). The decrease may extend up to 5 orders of magnitude (not shown) and finally leads to the low level of membrane conductance, which may be attributed to the conductance of the pure (unmodified) lipid bilayer. The effect is strongly dependent on the dose rate applied. A reduction of the dose rate leads to a decrease of the characteristic inactivation dose, D_{37}, which is defined as the radiation dose for which $I = I_0/e$ (e = Euler's number).

The range of dose-rate values was extended to 4–5 orders of magnitude by application of the method of pulse radiolysis in addition to the x-ray studies. The data in Fig. 2 suggest a linear relation (slope $\approx$ 1) between the sensitivity of the channels (expressed by $1/D_{37}$) and the inverse square root, $\dot{D}^{-1/2}$, of the dose rate. The relevance of this finding for the interpretation of the effect is discussed below. The D_{37}-dose was found to vary by a factor of 330 within the applied range of dose rates.

The effects of ionizing radiation on living matter are usually divided into *direct* and *indirect radiation effects*. In the former case, radiation is absorbed directly by the molecule considered, while in the latter case the effect is produced by free radicals generated by radiolysis of other molecular species (mainly water). Inactivation of polyene channels is clearly an indirect radiation effect. A theoretical argument as well as a series of experimental findings are in support of this conclusion:

1) Hit theory may be applied to estimate the D_{37}-dose (by the direct effect) for the molecular species in question. For a molecule with the mass μ, D_{37} (direct) is obtained from the equation (see, for example, (13))

$$D_{37}\,(\text{direct}) = W_H/\mu, \qquad (1)$$

with W_H = mean hit energy ($W_H \approx 60$ eV for biological material). Assuming a molar mass $M = L\mu$ (L = Avogadro's constant) of 10^4 g/mol (this is the order of magnitude estimated from the

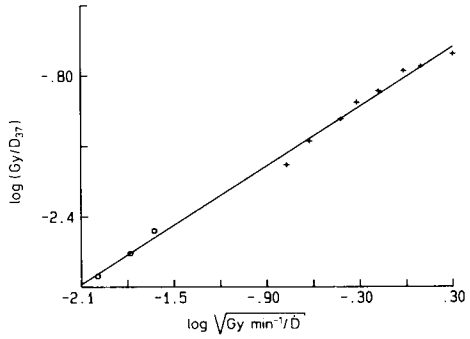

FIGURE 2 Radiation sensitivity as a function of the dose rate. The inverse of the characteristic radiation dose, $1/D_{37}$, is plotted as a function of the inverse of the square root of the dose rate, $1/\dot{D}^{1/2}$. The data were obtained from inactivation curves such as shown in Fig. 1. They refer to membranes formed from dioleoyllecithin (1%) and cholesterol (0.8%) in n-decane in the presence of 1–3 μg nystatin in the aqueous phases (0.1 M NaCl, pH 3 unbuffered, air-saturated). The data at comparatively small values of the dose rate, (+), refer to irradiation by 80 kV x-rays, those at comparatively large dose rates, (○), were obtained by irradiation of the membrane and its aqueous environment by sequences of pulses of 14 MeV electrons (pulse length 10 ns, repetition time 40 ms) supplied by the linear accelerator (LINAC) of the Hahn-Meitner-Institut. The average dose rate (including the repetition time) is plotted in this case. The solid line obtained by a least square fit corresponds to the equation $\log (Gy/D_{37}) = 1.13 \log (Gy\ min^{-1}/\dot{D})^{1/2} - 0.81$.

currently discussed channel structure, see Discussion), D_{37} (direct) $\approx 6 \times 10^5$ Gy is obtained from Eq. 1. This is many orders of magnitude larger than the D_{37}-values reported in the present publication (a few cGy = 10^{-2} Gy up to about 500 Gy).

2) A further important argument in favor of an indirect radiation effect may be derived from experiments in the presence of radical scavengers. The argument is based on the observation that the characteristic radiation dose, D_{37}, is increased in the presence of compounds which show high reactivity towards free radicals of water radiolysis. The compounds, acting as radical scavengers, exert a protective effect on the channels.

The primary radicals of water radiolysis—generated by ionization and by dissociation of water molecules—are the hydroxyl radical $OH^\bullet$, the hydrogen atom $H^\bullet$, and the hydrated electron e^-_{aq} (see, for example (14, 15)). In the presence of oxygen, the hydrated electron and the $H^\bullet$-radical are converted to the secondary superoxide radical $O_2^{-\bullet}$ and to the perhydroxyl radical $HO_2^\bullet$. The two oxygen radicals are in a pH-dependent equilibrium:

$$HO_2^\bullet \rightleftharpoons O_2^{-\bullet} + H^+. \qquad (2)$$

The pK_a of the weak acid HO_2 is 4.8, so that at pH < 4.8 the perhydroxyl radicals and at pH > 4.8 the superoxide radicals are predominant. The two oxygen radicals also play a role in the normal cell metabolism (i.e., in the absence of irradiation) and have been suggested to represent a major factor of oxygen toxicity in biological systems (10).

The main radical species present in air-saturated solutions are the $OH^\bullet$ radical and the oxygen species $HO_2^\bullet/O_2^{-\bullet}$. Both types of radicals contribute to the radiation

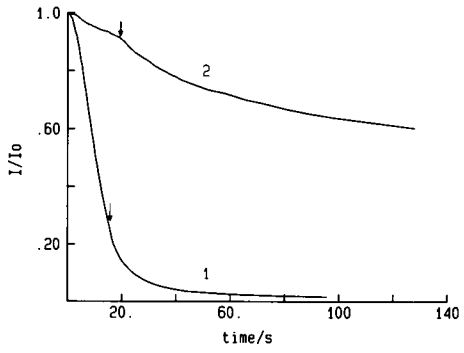

FIGURE 3 Inactivation of ion channels formed by nystatin at different oxygen concentrations. The membranes (formed from 1% dioleoyllecithin and 0.8% cholesterol) and their aqueous environment (containing 0.1 M NaCl/pH 3) were irradiated by pulses of 14 MeV electrons from time $t = 0$ up to the times indicated by arrows. (1): air saturated solution; total dose $D = 960$ Gy. (2): the cuvette used for membrane formation was flushed with argon to reduce the oxygen concentration by one to two orders of magnitude (total dose $D = 1250$ Gy).

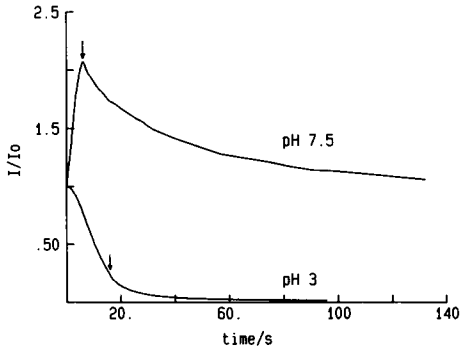

FIGURE 4 pH-dependence of the radiation effect. Membranes were irradiated by pulses of 14 MeV electrons up to times indicated by arrows. The experimental conditions were as described in the legend to Fig. 3.

effect considered. This may be deduced from an increase of the D_{37}-dose, which is observed in the presence of *t*-butanol, formate, or hydrogen peroxide (to be published in detail in a forthcoming publication).

The functional dependence of $1/D_{37}$ on the dose rate (see legend to Fig. 2) is explained on the basis of a radical chain mechanism (see Discussion). The mechanism of inactivation resembles in many respects the phenomenon of radiation-induced lipid peroxidation. The radical chain mechanism is initiated by $OH^\bullet$-radicals (or by secondary radicals derived from this species), and is augmented by oxygen radicals $O_2^{-\bullet}/HO_2^\bullet$. The kinetic chain length, v, which is introduced to describe the efficiency of peroxidation of the polyene antibiotics, is inversely proportional to the square root of the dose rate, $\dot{D}$ (see Eq. 8).

The important role of oxygen in the inactivation process—via oxygen radicals $O_2^{-\bullet}/HO_2^\bullet$ and via the suggested peroxidation process—is apparent from Fig. 3. A pronounced decrease of the rate of inactivation is observed, if the oxygen concentration in the system is reduced by one to two orders of magnitude. Fig. 3 also shows that the inactivation process—after initiation by irradiation for about 20 s—continues in the absence of irradiation. Inactivation may extend over about half an hour after irradiation (not shown). This is a time range which is several orders of magnitude larger than the lifetime of the initiating radical species. The phenomenon may be explained, assuming that the radical chain mechanism continues after irradiation is stopped (i.e., after the initiating radicals have disappeared).

The response of the membrane conductance at an irradiation was found to depend on the pH of the aqueous solution (see Fig. 4). A continuous inactivation after the start of irradiation was observed at pH 3 (see Fig. 1). This holds irrespective of whether the membrane is irradiated by 80 kV x-rays or by pulses of 14 MeV electrons. At pH 7.5, however, the behavior of the conductance was found to depend on the kind of irradiation.

While at comparatively small dose rates (x-ray experiments) a continuous inactivation was observed, there was a transient conductance increase followed by a delayed conductance decrease, if the membrane was irradiated by pulses of electrons of comparatively high dose rate.

There is no definite explanation for the pH-dependence of the radiation effect at present. It might possibly be caused by the presence of different species of oxygen radicals. The perhydroxyl radical $HO_2^{\bullet}$ is predominant at pH 3, while the superoxide radical $O_2^{-\bullet}$ is the prevalent species at pH 7.5. Independent of the pH, however, inactivation is the more important radiation effect. This is clearly evident from the x-ray experiments. Only inactivation was observed in this case, i.e., the transient conductance increase is buried within the more important inactivation process. It is only under the conditions of the very high dose rates of pulse radiolysis (and at comparatively high pH values) that the transient increase can be observed.

Further experimental results (to be presented in detail elsewhere) are as follows:

1) The D_{37}-dose of inactivation decreases with the degree of unsaturation of the fatty acid residues of the lipid used for membrane formation. The phenomenon may be understood via a participation of unsaturated fatty acid residues in the radical chain mechanism suggested to cause inactivation (see Discussion).
2) The sensitivity of nystatin channels is about twice that found for amphotericin B. This is in line with the well-known experience of an increased susceptibility of allylic H-atoms for abstraction by $OH^{\bullet}$ radicals. Hydrogen atoms of the methylene groups between the diene and the tetraene of the lactone ring of nystatin presumably represent the most vulnerable positions when attacked by radicals.
3) The D_{37}-dose was found to decrease with increasing concentration of NaCl in the aqueous solution. The mean lifetime of highly reactive primary $OH^{\bullet}$ radicals is extremely short. In aqueous NaCl solutions (i.e., in the presence of Cl^- ions), part of $OH^{\bullet}$ radicals is converted to secondary $Cl_2^{-\bullet}$ radicals of considerably longer lifetime (16–18). As a consequence, the radical-induced modification of polyene molecules seems to proceed with higher efficiency. A further decrease of the D_{37}-values was observed, if NaCl was replaced by NaBr. The higher reactivity of the $Br_2^{-\bullet}$ radical was already discussed in the context of radiation-induced lipid peroxidation (detected via an increased membrane capacitance) (19).

The great sensitivity of polyene channels at high concentrations of NaCl and NaBr (1 M) and at sufficiently small dose rates ($\dot{D} \approx 0.01$ Gy/min) is illustrated by the following data. D_{37}-doses of the order of a few cGy (i.e., 9 and 3.4 cGy, respectively) were observed under these conditions.

DISCUSSION

The polyene antibiotics nystatin and amphotericin B show a series of biological effects such as antifungal, antiviral, and antitumor activity. Amphotericin B is widely used as a drug to treat systemic fungal infections. It is generally thought that the compounds act at the membrane level. Their influence on the properties of cellular membranes has been the subject of numerous studies (for a review, see (20, 21)).

An important property of nystatin and of amphotericin B is their ability to form ion channels in sterol-containing biological membranes or in artificial lipid membranes. The conductance of planar lipid membranes, separating two salt solutions, is increased by many orders of magnitude, if submicromolar concentrations of amphotericin B (or micromolar concentrations of nystatin) are added to the aqueous phase (for a review of early experiments see (22)). A strong enhancement of the membrane permeability for monovalent cations or anions (with a small preference for anions) is responsible for this effect. The phenomena have been interpreted on the basis of aqueous channels formed by aggregates of polyene antibiotics and sterol molecules (22, 23). Each aggregate has been suggested to consist of two "barrels" hydrogen-bonded end to end. The barrels are assumed to be formed by 8–10 amphotericin B (nystatin) monomers arranged circumferentially as staves and stabilized by sterol molecules. Single barrels, formed by one-sided addition of polyenes, are also active, though only at comparatively thin lipid bilayers and at higher antibiotics concentration. The pore-like nature of the conductance induced by polyene antibiotics was confirmed by single-channel measurements (24–26).

The conductance induced by polyene channels in planar lipid membranes is reduced to virtually zero after irradiation of the membrane and its aqueous environment by ionizing radiation (see Results section). The inactivation curves show the strange phenomenon of an *inverse dose-rate effect.* This term has been introduced in the literature to describe radiation effects which show an increasing sensitivity with decreasing dose rate (at an equal radiation dose applied). The *normal dose-rate effect,* which has been frequently observed in the field of radiation biology, shows an increasing radiation effect at increasing dose rate.

Inverse dose-rate effects have been observed at the cellular level in the form of radiation-induced neoplastic transformations (27, 28) or mutations (29). At the molecular level, radiation-induced (30–34) or chemically-induced (35) lipid peroxidation has been found to show a pronounced inverse dose-rate behavior (see (5) for a review on this subject). The present paper reports about the first observation of an inverse dose-rate effect at the level of ion channels in membranes.

There seems to be a rather close relationship of the mechanisms of radiation-induced lipid peroxidation and of radiation-induced inactivation of polyene channels. The relationship is based on the following experimental findings:

1) Both phenomena seem to depend on the presence of the same kind of free radicals, $OH^{\bullet}$ and $HO_2^{\bullet}/O_2^{-\bullet}$.
2) The two phenomena are strictly dependent on the presence of oxygen.
3) The extent of radiation-induced lipid peroxidation as well as that of channel inactivation (D_{37}-dose) was found to be inversely proportional to the square root of the dose rate, $\dot{D}$.

Lipid peroxidation is usually interpreted on the basis of a radical chain mechanism, which explains all three findings and which (in its simplest form) may be written as follows:
Initiation (by a radical species $X^{\bullet}$, e.g., $OH^{\bullet}$):

$$LH + X^{\bullet} \xrightarrow{k_i} L^{\bullet} + XH, \tag{3}$$

Propagation of the chain:

$$L^{\bullet} + O_2 \xrightarrow{k_0} LOO^{\bullet} \tag{4}$$

$$LOO^{\bullet} + LH \xrightarrow{k_p} LOOH + L^{\bullet}, \tag{5}$$

Termination of the chain:

$$LOO^{\bullet} + LOO^{\bullet} \xrightarrow{k_t} \text{nonradical product.} \tag{6}$$

The reaction chain is initiated by reaction of a radical species $X^{\bullet}$ with a lipid molecule LH. The chain propagates via formation of peroxyl radicals $LOO^{\bullet}$ which—by abstraction of H atoms—form stable lipid hydroperoxides at the expense of further lipid radicals $L^{\bullet}$. Thus, the peroxidation process continues until the radical species are eliminated by radical–radical interactions (e.g., Eq. 6). Eqs. 3–6 predict an inverse dose-rate behavior. The length of the radical chain is limited by the bimolecular termination reaction. Its importance increases with the second power of the radical concentration $LOO^{\bullet}$, which depends linearly on the dose rate, $\dot{D}$.

The reaction chain may be analyzed by introducing the *kinetic chain length, v*, defined by

$$v = \frac{\text{number of } O_2 \text{ molecules consumed}}{\text{initiating radical } X^{\bullet}} \tag{7}$$

v is equal to the number of lipid hydroperoxides formed per initiating radical. The formal analysis of the reaction scheme yields (assuming $v > 1$, see (5) or (35)):

$$v = \frac{o\mathrm{LH}}{(\alpha\dot{D})^{1/2}}, \tag{8}$$

with $o = k_p/(2k_t)^{1/2}$, LH = concentration of the lipid, αD = formation rate of radicals $X^{\bullet}$.

Eq. (8) predicts the same dose rate dependence for the kinetic chain length, v, as found experimentally for the D_{37}-dose of channel inactivation (see Fig. 2). We think that channel inactivation is the result of a peroxidation process of the polyene antibiotics. Further support for this conclusion is obtained from the following arguments:

1) The lactone ring of amphotericin B contains a heptaene, that of nystatin a tetraene and a diene. In this respect the two molecules resemble highly unsaturated fatty acid residues of lipid molecules, which are well-known for their susceptibility to peroxidation. Similar to unsaturated lipids, the polyene antibiotics show the phenomenon of autoxidation (21, 36).
2) The D_{37}-dose is decreased, if the channels are incorporated into a lipid membrane with a higher content of double bonds. This may be understood on the basis of an increased kinetic chain length in the presence of lipids with a higher degree of unsaturation. The radical chain seems to comprise both, lipid molecules and polyene antibiotics. The radiation effect is, however, also present in the absence of oxidizable fatty acid residues (membranes formed from diphytanoyllecithin), though at larger D_{37}-values.

Eqs. 3–6 certainly represent only a minimum model for the channel inactivation. The model does not include the oxygen radicals $O_2^{-\bullet}/HO_2^{\bullet}$. Their role—as in the case of lipid peroxidation—might consist in an elongation of the radical chain by decomposing lipid hydroperoxides LOOH to alkoxy radicals $LO^{\bullet}$ (see (5) for a discussion of this controversial problem).

In summary, the high sensitivity towards ionizing radiation of ion channels formed by the polyene antibiotics amphotericin B and nystatin may be explained by assuming a peroxidation process initiated by free radicals of water radiolysis. The polyene chains of the lactone rings —according to present ideas about the channel structure (22, 23, 26)—are believed to represent the hydrophobic exterior of the channel. Peroxidation of this part of the molecule could destabilize the barrel-like structure.

The general structural principle of ion channels consists in a hydrophobic exterior surface in contact with the lipid phase and a hydrophilic inner surface exposed to water and ions. The radiation-induced peroxidation process may reduce the hydrophobic character of the exterior surface, i.e., may modify the energy balance of the channel structure. This should have consequences for the probability and lifetime of open channels. So far, the study has concentrated on providing a mechanistic interpretation of the inverse dose-rate behavior. The existence of such a behavior, which forms the basis of substantial effects at very low doses of ionizing radiation, is reported for the first time at the level of ion channels.

The effect of radiation on the single-channel properties will be a matter of future investigations.

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