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Topical Review

Functional Consequences of Oxidative Membrane Damage

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Abstract. The interaction of reactive oxygen species with biological membranes is known to produce a great variety of different functional modifications. Part of these modifications may be classified as *direct effects*. They are due to direct interaction of the reactive species with the molecular machinery under study with a subsequent chemical and functional modification of these molecules. An important part of the observed functional modifications are, however, *indirect effects*. They are the consequence of an oxidative modification of the environment of biological macromolecules. Lipid peroxidation—via its generation of chemically reactive products—contributes to the loss of cellular functions through the inactivation of membrane enzymes and even of cytoplasmic (i.e., water soluble) proteins. Oxidation of membrane lipids may, however, also increase the efficiency of membrane functions. This was observed for a series of transport systems. Lipid peroxidation was accompanied by activation of certain types of ion channels and ion carriers. The effect is due to an increase of the polarity of the membrane interior by accumulation of polar oxidation products. The concomitant change of the dielectric constant, which may be detected via the increase of the membrane capacitance, facilitates the opening of membrane channels and lowers the inner membrane barrier for the movement of ions across the membrane. The predominant effect, however, at least at a greater extent of lipid peroxidation, is the inhibition of membrane functions. The strong increase of the leak conductance contributes to the depolarization of the membrane potential, it destroys the barrier properties of the membrane and it may finally lead, via an increase of cytoplasmic Ca^{2+} concentration, to cell death. The conclusions were derived from experiments performed with different systems: model systems in planar lipid membranes, native ion channels either reconstituted in lipid membranes or investigated in their natural environment by the patch-clamp method, and two important ion pumps, the Na/K-ATPase and the sarcoplasmic reticulum (SR) Ca-ATPase.

Key words: Reactive oxygen species — Free radicals — Lipid peroxidation — Ion channels — Ion pumps — Membrane potential

Introduction

The structural modifications and their functional consequences induced by reactive oxygen species (ROS) include a great variety of different cellular constituents and phenomena. Though the genetic substance is of primary importance, cellular membranes also seem to play a central role in this respect. Biological membranes control the concentration of cellular metabolites in the cytoplasm and in cellular organelles by highly selective passive and active transport phenomena. They are indispensable, in the form of mitochondria and of the thylakoid membranes of chloroplasts, for the biological energy supply. Biological membranes also participate in the intra- and intercellular communication and, in the form of action potentials, they control the transfer of information between different organs such as nerves and muscles. As a result of these manifold tasks, an oxidative modification of membranes will have great consequences for a biological organism. Oxidative membrane damage has been found to be of great pathological importance and has been discussed in relation with the phenomena of ischemia/reperfusion (and the pathogenesis of myocardial and cerebral tissue injury) and of central nervous system trauma and shock [10, 11, 37]. In addition, numerous studies have shown that lipid peroxidation seems to be involved in the pathogenesis of various degenerative

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diseases including arteriosclerosis, diabetes, inflammation, and neurodegenerative disorders.

On the molecular level, which is considered here, the importance of oxidative membrane damage is twofold. On the one hand, there are the manifold membrane functions, which may be modified by direct attack of the reactive species on the membrane components that are responsible for the functions. However, one has to consider also the indirect consequences of oxidative membrane damage. Lipid peroxidation modifies the environment of not only \membrane proteins and may in this way influence their functional efficiency. In addition, highly reactive products of lipid peroxidation are able to react with and to modify the structure and function of membrane proteins but also of nucleic acids and of cytoplasmic enzymes [70, 112]. Therefore, two different ways of oxidative modifications of cellular constituents have to be considered in general: the direct modification by reactive oxygen species and the indirect modification via reactive products of lipid peroxidation.

The present review mainly concentrates on functional modifications of membrane transport in the form of ion channels and ion pumps. It is, however, not intended to provide a complete survey about the manifold channel and pump types and of other transport pathways. Kourie's review [59] should be consulted in this respect. The approach considered here concentrates on a few systems, which were selected to elaborate important principles. Model systems in planar lipid membranes provided detailed information on some fundamental mechanisms of functional modifications. These studies were supplemented by patch-clamp investigations on cellular ion channels. Both, direct inactivation by ROS and indirect effects originating from lipid peroxidation were observed, which sometimes act in parallel and may lead to activation of membrane transport followed by inactivation.

Lipid peroxidation shows the strange phenomenon of a so-called inverse dose-rate behavior (*see* [98] for a review). This means that high production rates of free radicals, which initiate lipid peroxidation, yield a smaller amount of reactive end products than low production rates of free radicals (at equal total amounts, i.e., at equal "dose", of initiating species in both cases). This is a consequence of the radical chain mechanism underlying lipid peroxidation. The term "inverse dose-rate effect" was originally introduced in the field of radiation biology. It is distinguished from the normal dose-rate effect, where high dose-rates are more effective than low dose-rates. Inverse dose-rate effects may lead to an underestimation of the risks associated with the application of ionizing radiation. In the case of the systems considered here, the observation of an inverse dose-rate behavior is used to distinguish between a direct effect of free radicals and an indirect effect via products of lipid peroxidation. This is justified by the fact that usually only the indirect effect is expected to show an inverse dose-rate behavior. An exception to this rule are molecular systems with a (partially, at least) structural similarity to unsaturated fatty acid residues (such as the polyene antibiotics discussed in the section Inactivation of Polyene Channels by a Radical Chain Mechanism). In this case, peroxidation initiated by direct interaction with reactive species can give rise to an inverse dose rate behavior.

Table 1. Reactive oxygen species[38]

Radicals	Nonradicals
Superoxide, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\cdot}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\cdot}$	Ozone, O_3
Hydroperoxyl, $HO_2^{\cdot}$	Singlet oxygen, $^1\Delta g$
Alkoxyl, $RO^{\cdot}$	Peroxynitrite, $ONOO^-$

While the inverse dose-rate effect at free radical-induced lipid peroxidation is well established, the observation of such phenomena at the functional level of membrane transport and at the level of enzyme inactivation have been described only in recent years. The phenomena will be summarized and discussed in the present review.

Reactive Oxygen Species and Their Generation

Oxidative damage is due to the action of oxygen radicals, such as the hydroxyl radical $OH^{\cdot}$, and the superoxide radical $O_2^{\cdot -}$, but is also due to some nonradical derivatives of oxygen, such as singlet oxygen $^1O_2$ or hydrogen peroxide H_2O_2 (*see* Table 1). In the laboratory, a number of different procedures have been applied to study oxidative damage [38]. The Fenton reaction or Fenton-like reactions may be used to generate $OH^{\cdot}$ radicals by interaction of iron or iron chelates with peroxides. Several enzymes such as xanthine oxidase have been found to reduce O_2 to $O_2^{\cdot -}$. Water-soluble or lipid-soluble azo initiators can be used to generate $RO_2^{\cdot}$ radicals (and $O_2^{\cdot -}$ radicals) for studies of lipid peroxidation and antioxidant activity. Two further methods, which have been very useful throughout the study of oxidative membrane damage, will be briefly discussed, namely the generation of hydroxyl and superoxide radicals by the method of water radiolysis and the generation of singlet oxygen by the photodynamic method.

The Radiolysis of Water

Absorption of ionizing radiation in water leads to the formation of the primary radicals $OH^{\cdot}$, $H^{\cdot}$, and e_{aq}^- (apart from some other nonradical species [16, 92,

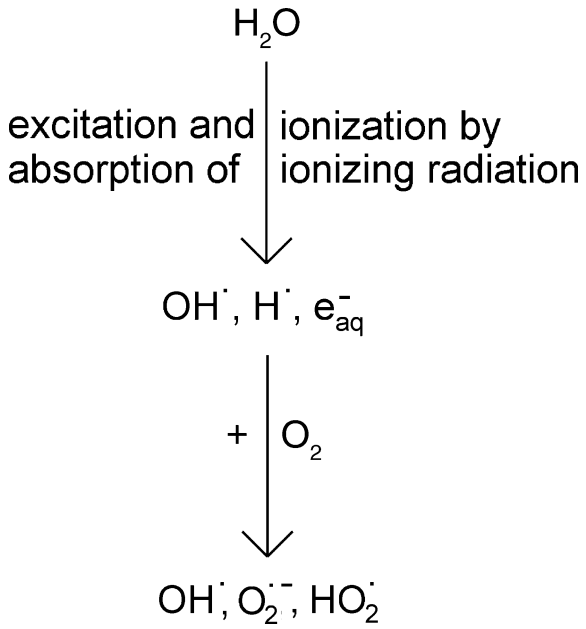

Fig. 1. Free radical formation by water radiolysis in the absence and presence of oxygen.

98]). In the presence of oxygen, the hydrogen radical $H^{\cdot}$ and the hydrated electron e_{aq}^{-} react with O_2 and form the hydroperoxyl radical $HO_2^{\cdot}$ and the superoxide radical $O_2^{\cdot-}$ (Fig. 1). The two oxygen radicals $HO_2^{\cdot}$ and $O_2^{\cdot-}$ are in a pH-dependent equilibrium:

$$HO_2^{\cdot} \rightleftarrows O_2^{\cdot-} + H^+ \qquad (1)$$

The pK of the weak acid $HO_2^{\cdot-}$ is 4.8, so that at $pH \ll 4{,}8$ the hydroperoxyl radical and at $pH \gg 4.8$ the superoxide radical is predominant. As a consequence, the hydroxyl $OH^{\cdot}$ and the superoxide radical $O_2^{\cdot-}$ are the main oxidizing species of water radiolysis at pH 7. At pH 3, $O_2^{\cdot-}$ is replaced by its protonated, and more reactive form $HO_2^{\cdot}$. The action of $OH^{\cdot}$ radicals and of the conjugated pair $O_2^{\cdot-}/HO_2^{\cdot}$ may be distinguished from one another by using radical scavengers.

The method of water radiolysis has several advantages compared with other methods. The radiation chemistry of water has been studied over several decades. As a consequence, the products of water radiolysis and their concentrations are known in great detail. The concentration of a radical R is determined by its G_R-value, which is defined as the number of radicals R generated by 100 eV of absorbed radiation energy. The G_{OH}-value for $OH^{\cdot}$ is 2.7. For the conjugated pair $O_2^{\cdot-}/HO_2^{\cdot}$ (in air-saturated water) $G(O_2^{\cdot-}/HO_2^{\cdot}) = 3.2$ was found. Using these values, a simple calculation yields a concentration of $OH^{\cdot}$ -radicals of 2.8 μM and of the conjugated pair $O_2^{\cdot-}/HO_2^{\cdot}$ of 3.3 μM, if air-saturated water is exposed

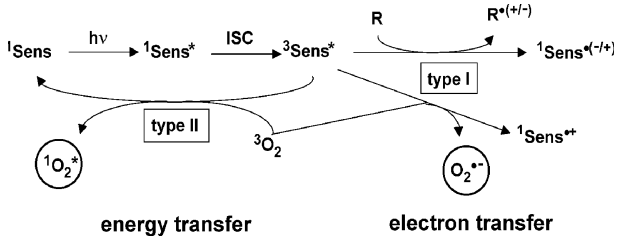

Fig. 2. Photodynamic generation of reactive oxygen species. Sensitizer molecules in the singlet ground state ^{1}Sens are transferred into the excited state ^{1}Sens* by absorption of light (energy $h\nu$) and from there by intersystem crossing into the triplet state ^{3}Sens*. ^{3}Sens* reacts with O_2 by energy transfer or alternatively by electron transfer to form singlet oxygen $^1O_2$ or the superoxide radical $O_2^{\cdot-}$.

to a dose of 1 Gy = 1 J/kg by using radiation of low linear energy transfer (X-rays, γ-rays or fast electrons). In the author's laboratory a conventional X-ray machine has been applied to irradiate membranes or proteins and their respective aqueous environments with dose rates up to 100 Gy/min [98].

A further advantage of the method of water radiolysis is the possibility of studying the kinetics of radical reactions by using short pulses of ionizing radiation from a linear accelerator (pulse radiolysis).

The Photodynamic Method

Oxidation of biological macromolecules and of cellular structures may also be induced by visible light, however, in the presence of photosensitizers and of oxygen. Under these conditions illumination causes a multitude of functional changes in biological systems, usually summarized as photodynamic effects [29, 104]. The light energy absorbed by the photosensitizer is able to modify nearly all types of biologically relevant macromolecules via redox or energy-transfer reactions (Fig. 2). Photodynamic effects finally lead to cell death preferentially by apoptosis (*see* citations in [22, 72]). This is applied throughout a series of clinical applications such as the photodynamic therapy (PDT), where the absorption of visible light by an appropriate sensitizer is used for tumor destruction [21, 22].

In many cases, photodynamic effects have been found to be due to the generation of the reactive oxygen species $^1O_2$ and $O_2^{\cdot-}$. This holds especially for singlet oxygen, which has been found to interact with many cellular targets [29, 72, 74, 104]. In the case of biological membranes, an important consequence of singlet oxygen formation is lipid peroxidation [34–36].

The two species $^1O_2$ and $O_2^{\cdot-}$ are formed by energy transfer and by electron transfer from the triplet state of the sensitizer molecule (*see* Fig. 2). The two types of reactions are usually characterized as type II and type I, respectively, though this classification is inconsistently used in the literature [29, 30, 104].

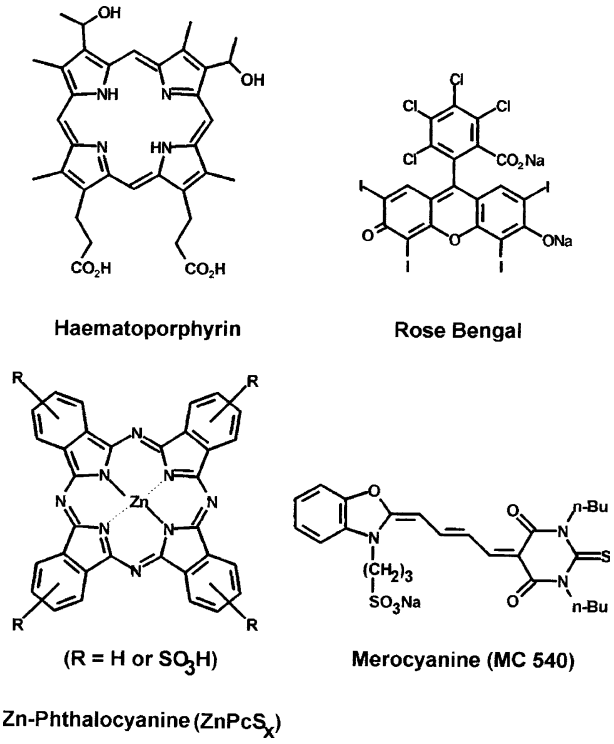

Fig. 3. Important membrane-active photosensitizers.

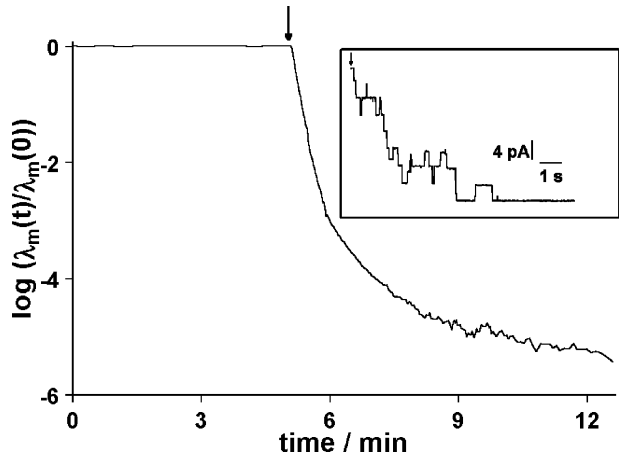

Fig. 4. The conductance $\lambda_m(t)$ (normalized to the initial conductance $\lambda_m(0)$) of a lipid membrane in the presence of gramicidin A and of the photosensitizer rose bengal. The arrow indicates the start of illumination. $\lambda_m(0)$ corresponds to the conductance of about 2 million gramicidin channels (*see* [60] for further experimental details). The final part of the inactivation curve was detected at a strongly enhanced sensitivity of current measurement to illustrate photodynamic inactivation at the level of single ion channels (*see* inset).

Some important photosensitizers, which have been found to produce oxidative membrane damage, are shown in Fig. 3. A mixture of derivatives of haematoporphyrin, in the form of photofrin II, is frequently applied throughout photodynamic therapy (PDT) [8, 21, 22]. Cellular membranes such as mitochondria, lysosomes and the plasma membrane have been evaluated as potential sites of action for a series of different sensitizers used in PDT [22, 77]. The xanthene dye rose bengal is well-known for its comparatively high yield of singlet oxygen [73]. Rose bengal, Zn-phthalocyanine and merocyanine 540 (as well as other sensitizers) have been characterized as very efficient in their ability to produce light-induced modifications of membrane functions [4, 66, 90, 95, 113, 116].

Direct and Indirect Consequences of the Interaction of Reactive Oxygen Species with Biological Membranes

As mentioned in the Introduction, modifications of membrane functions may be induced by direct interaction of the reactive species with the molecular machinery considered and/or by an oxidative modification of its environment. The two alternatives are usually difficult to distinguish in practice. Distinction is facilitated if the system can be incorporated into artificial lipid membranes. In this case, the oxidizability of the environment can be varied by selection of appropriate lipids. This is shown by considering ion transport through two well-known model channels formed by the peptide gramicidin A and by polyene antibiotics.

Inactivation of ion Channels Formed by Gramicidin A

Gramicidin A forms highly selective pathways across biological membranes for monovalent cations. The electric membrane current induced by a constant electric field in the presence of small concentrations of the peptide shows the typical stepwise fluctuations usually associated with the opening and closing of ion channels. Details of the channel structure and function have been studied and reviewed extensively [15, 18, 39, 47, 53, 121, 122]. The hydrophobic, linear pentadecapeptide with tryptophan residues at positions 9, 11, 13 and 15 forms helical structures. Dimers, generated by head to head association of two of the helical monomers, are assumed to bridge the hydrophobic, bimolecular lipid phase of biological membranes, and to allow ion permeation through a central, hydrophilic opening.

Gramicidin channels were found to react very sensitively if exposed to ionizing radiation (radiolysis [2, 60, 97, 99, 101]), to UV light (photolysis [13, 14, 44, 101]) or to visible light in the presence of membrane-active photosensitizers and oxygen (photodynamic inactivation [60, 84–86, 93, 103]. Figure 4 illustrates the potential of the lipid bilayer technique for this kind of studies. The magnitude of the constant electric current before illumination of the

membrane indicates the simultaneous activity of about 2 million gramicidin channels. Following illumination, the electric current decays over about 6 orders of magnitude within a few minutes, until the current fluctuations indicate the presence of only a few active channels, which successively disappear to show virtually zero current (*see* inset of the figure). Starting with the situation of a macroscopic multichannel system and finally ending with the microscopic system of single ion channels, the complete inactivation behavior can be observed in a single experiment. The detailed analysis of the data showed that most of the gramicidin channels were completely and irreversibly inactivated. Only a small percentage of the single-channel events indicated partially damaged ion channels [60].

Studies performed by different groups led to the result that for all three inactivation procedures, i.e., radiolysis, photolysis and photodynamic inactivation, modifications of the tryptophan residues are of central importance for channel inactivation. Replacement of the tryptophan residues by other aromatic amino acids strongly reduced the sensitivity of the ion channels [99, 101, 103]. The time dependence of the loss of channel function during UV irradiation was similar to the decay of the tryptophan fluorescence of gramicidin A [44]. Mass spectrometry of photodynamically treated gramicidin A was accompanied by oxidation and fragmentation of the peptide at the positions of the tryptophan residues [60]. The special importance of the tryptophan residues was further emphasized by the finding that their chemical modification by N-bromosuccinimide reduced the fluorescence and the conductance of gramicidin channels and of colicin E1 [93].

We will now deal with the question, how modification of the tryptophan residues is brought about. A series of different approaches showed that inactivation is due to direct interaction of the reactive species, generated by the respective method, with the tryptophan residues of gramicidin A, i.e., by combined action of the free radicals $OH^{\cdot}$ and $HO_2^{\cdot}$ (water radiolysis [99]), by direct absorption of UV-light (photolysis [44]), and by $^1O_2$ (photodynamic inactivation [85]). Indirect inactivation by products of lipid peroxidation can be largely excluded, since the sensitivity of gramicidin channels decreases with increasing oxidizability of the lipid used for membrane formation (Fig. 5). Larger radiation doses have to be applied at radiolytic inactivation if the number of double bonds of the fatty acid residues of the lipid is enhanced. The highest sensitivity is found if the membrane is formed by the completely saturated lipid diphytanoylphosphatidylcholine (curve *1* of Fig. 5). The tryptophan residues of gramicidin A seem to be protected against the attack of $OH^{\cdot}$ radicals by the double bonds of the lipids, which on the other hand, are responsible for the initiation of lipid

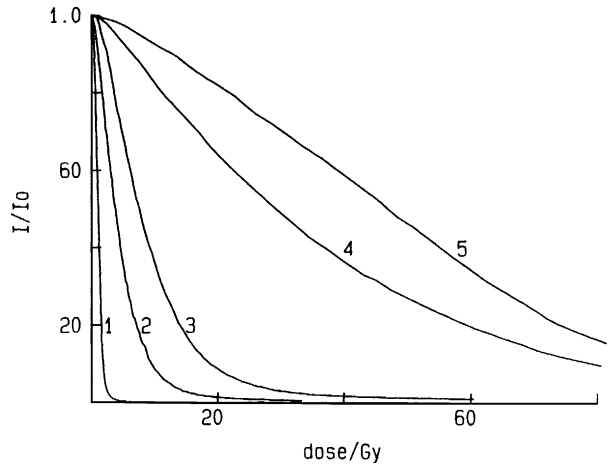

Fig. 5. Inactivation by 80 kV X-rays of ion channels formed by gramicidin A in planar phosphatidylcholine membranes with different degrees of unsaturation [2]: (*1*) diphytanoylphosphatidylcholine, (*2*) dioleoylphosphatidylcholine, (*3*) dilinoleoyl-phosphatidylcholine, (*4*) 4:1 mixture of dilinolenoylphosphatidylcholine and diphytanoylphosphatidylcholine, (*5*) 7:3 mixture of diarachidonoyl-phosphatidylcholine and diphytanoylphosphatidylcholine. *I* is the current observed at a voltage of 50 mV, I_0 is the current before irradiation.

peroxidation. The data are most easily explained by a competition between lipid double bonds and tryptophan residues for $OH^{\cdot}$ radicals [2]. Similar effects, i.e., protection by lipid double bonds, were also reported for photodynamic inactivation of gramicidin [85].

Gramicidin A is usually considered a model channel, the structure and function of which have been investigated in great detail. This holds also for its mechanism of radiolytic, photolytic or photodynamic inactivation. Fragmentation of the peptide at its tryptophan residues interrupts the free pathway for cations across the lipid bilayer and allows a simple and straightforward understanding of channel inactivation [60]. The photosensitized rupture of peptide bonds, however, has been observed very rarely so far. Therefore the generalization of this finding for other ion channels requires further studies.

Inactivation of Polyene Channels by a Radical Chain Mechanism

The polyene antibiotic amphotericin B is frequently used as an antifungal agent for the treatment of systemic infections [9]. There is general agreement that this substance, as well as other polyene macrolide antibiotics, acts at the membrane level (*see* [6] for a review). An important property of amphotericin B (and of the related compound nystatin) is their ability to form ion channels in sterol-containing biological membranes, or in artificial lipid membranes. Polyene channels were found to react extremely sensitively if exposed to radiation-induced free radicals of water radiolysis [3, 124] or to visible light in the presence of

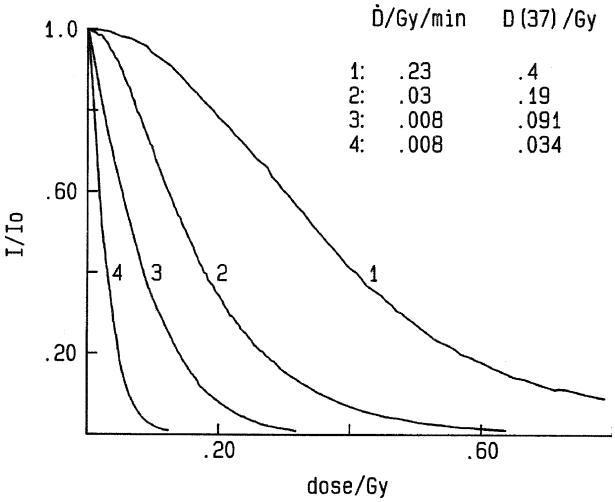

Fig. 6. Inactivation by 80 kV X-rays of polyene amphotericin B channels as a function of dose-rate [124]. The radiation-induced decay of the electric current, at a constant voltage of 50 mV, was followed as a function of radiation dose (I_0 = current before irradiation). Membranes were formed from solutions of 1% dioleoyllecithin and 0.2% cholesterol in n-decane. The aqueous solutions separated by the membrane contained 1 mol dm^{-3} NaCl (curves *1–3*) or 1 mol dm^{-3} NaBr (curve *4*) and 200–400 ng/ml amphotericin B (pH3, unbuffered). Reproduced from [124] with permission from Int. J. Rad. Biol., www.tandf.co.uk.

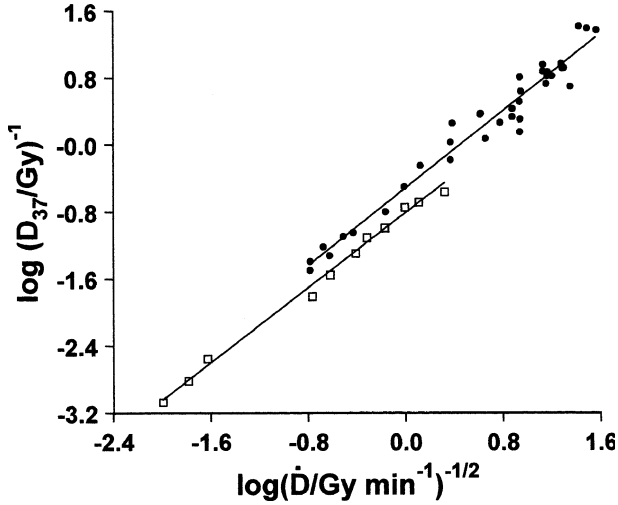

Fig. 7. Radiation sensitivity of amphotericin B (*filled circles*) and nystatin (*empty squares*) as a function of dose-rate. The logarithm of the inverse of the characteristic dose, $1/D_{37}$, was plotted as a function of the logarithm of the inverse of the square root of the dose-rate, $1/\dot{D}^{1/2}$. The data were obtained from inactivation curves such as shown in Fig. 6 under slightly different experimental conditions for the two polyene antibiotics (*see* references [3] and [124] for experimental details).

the photosensitizer rose bengal [55]. They represent another example of direct inactivation, though the phenomenon of lipid peroxidation plays an essential role in this case. Caused by the large number of double bonds, which form part of the structure of polyene antibiotics (6 in the case of nystatin and 7 in the case of amphotericin), the free radical-induced chain reaction, well-known from lipid peroxidation, may be expected to function also in a membrane environment of comparatively low oxidizability. This is especially true in view of the fact that the channels consist of aggregates of polyene molecules [20, 28, 48]. Each aggregate has been suggested to consist of one barrel, or of two barrels, hydrogen-bonded end to end. The barrels are formed by eight to ten polyene molecules arranged circumferentially as staves and stabilized by sterol molecules. The orientation of the lactone ring of amphotericin B is assumed in such a way in order to provide a hydrophobic exterior and a hydrophilic interior of the channel. As a consequence, the channel exterior forms a cluster of about 100 double bonds, which may be considered as extremely vulnerable towards free radical-induced peroxidation processes. Experimental evidence that a radical chain mechanism is involved at polyene channel inactivation was obtained from the observation of an inverse dose-rate behavior illustrated in Figs. 6 and 7. The sensitivity of the channels is strongly enhanced if the applied dose-rate decreased. The effect was observed over 7 orders of magnitude of the dose-rate in two different series of experiments (i.e., from 7×10^{-4} to 1×10^4 Gy/min). The effect may be quantified, if the D_{37}-dose (i.e., the dose required to decrease the initial membrane current to 37%, (i.e., to I_0/e, e = Euler's number) is used to characterize the channel sensitivity. A linear relation between the inverse of the characteristic dose, $1/D_{37}$, and the inverse of the square root of the dose rate, $1/\dot{D}^{1/2}$, was observed (Fig. 7), i.e., $1/D_{37} \sim \sqrt{\dot{D}}$ (with $\dot{D} = dD/dt$, dose rate; $D =$ dose; $t =$ time).

A similar relation is obtained as a direct consequence of a simple radical chain mechanism shown in Fig. 8*A* [3, 98, 124]. Eqs. (1)–(4) predict an inverse dose-rate behavior. The radical chain described by Eqs. (2) and (3) continues to proceed until it is terminated by Eq. (4). The importance of this bimolecular reaction increases with the second power of the radical concentration $LOO^{\cdot}$, i.e., with the second power of the dose rate. Therefore, the kinetic chain length v, i.e., the number of hydroperoxides LOOH formed per initiating radical $X^{\cdot}$ (or the number of propagation cycles), is smaller at higher dose rates applied. The quantitative analysis of the reaction scheme yields $v \sim 1/\sqrt{\dot{D}}$, which agrees with the relation obtained experimentally if the kinetic chain length v is assumed to be proportional to the sensitivity, $1/D_{37}$, of the polyene channels [3, 124].

The actual peroxidation process of lipids [24, 31–33, 45, 96, 98, 111] or of polyene antibiotics is considerably more complex than the simple reaction scheme which, however, is sufficient to describe the

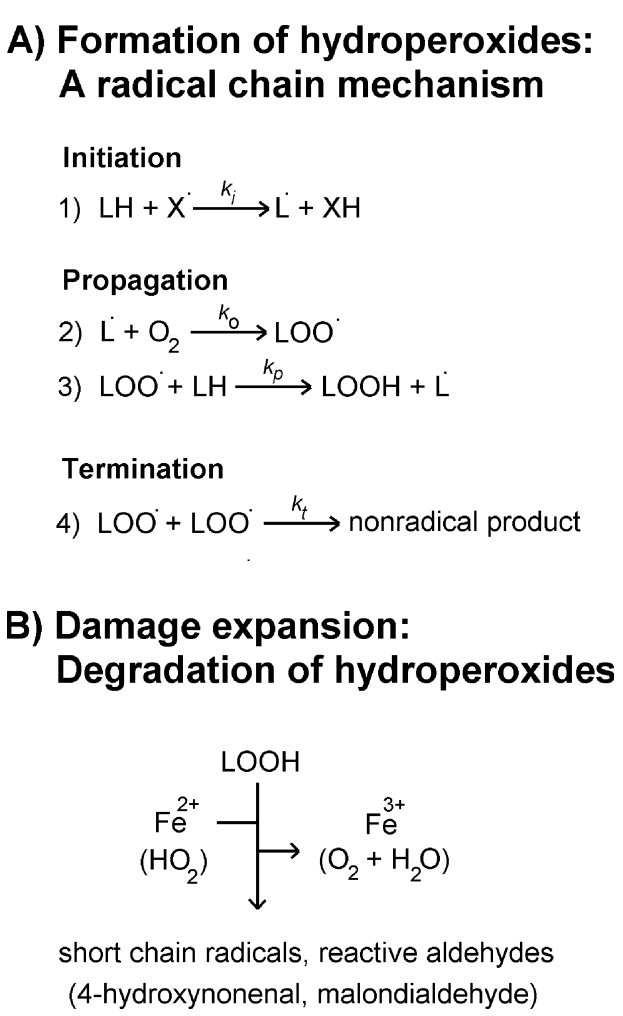

Fig. 8. Simplified reaction scheme of lipid peroxidation.

correct dose-rate dependence. The same inverse dose-rate behavior was found by different authors throughout their study of lipid peroxidation (*see* [98] for a review). Contrary to gramicidin channels, which are protected by unsaturated fatty acid residues of the lipids, an enhancement of the oxidizability of the lipids used for membrane formation gave rise to a decrease of the characteristic D_{37}-dose, i.e., to an increase of the sensitivity of polyene channels [124]. One may assume that both, polyene channels and lipids, participate in the same radical chain mechanism. As a consequence, the kinetic chain length of the combined mechanism is increased, if the amount of double bonds of the lipids is enhanced.

Further experiments indicated that the radical chain mechanism is initiated by $OH^{\cdot}$ radicals or by its secondary radicals $Cl_2^{-\cdot}$ and $Br_2^{-\cdot}$ formed in the presence of NaCl and NaBr, and that the peroxidation process is promoted by $HO_2^{\cdot}$ radicals [124]. Considering these findings in combination with the structure mentioned above, inactivation of polyene channels may be imagined to proceed as follows:

One of the characteristics of the open channel structure is the hydrophobic exterior and the hydrophilic interior. Peroxidation renders the lipid-like exterior of the channels less hydrophobic. This gives rise to a destabilization of the polyene aggregates, which finally leads to channel closure. Single-channel experiments indicated that the conductance of the open channel (as in the case of gramicidin A) is reduced to virtually zero as a consequence of a radiolytic or photodynamic inactivation [55, 124].

Activation of Membrane Transport: An Indirect Effect of Oxidative Membrane Damage

In the case of polyene antibiotics, peroxidation gives rise to direct inactivation of the corresponding ion channels. This must, however, be considered as a special case. Frequently, a given transport system is indirectly influenced by peroxidation of its membrane environment. Two different indirect effects of oxidative modification of the membrane have been observed, which act in an antagonistic way:

a) Inactivation phenomena, i.e., damage of membrane functions, which are generally observed at higher oxidative modifications, and which will be considered in the following section.
b) Activation of membrane transport usually observed at comparatively small oxidative modification of the membrane.

Activation of membrane transport has frequently been observed at the level of ion channels, which have been found to show transitions from an inactive to an active state if the membrane is exposed to oxidizing chemicals, to ionizing radiation, or after a photosensitized modification of the membrane. This holds for cellular ion channels [25–27, 41, 54, 63, 65, 88, 123] as well as for model channels in lipid bilayer membranes [50, 93, 125]. Activation was, however, also observed for ion carriers of the valinomycin type in lipid membranes [50, 100]. Therefore, activation is not a special property of ion channels, but seems to be a general phenomenon of membrane transport with a common physical basis. We found that activation seems to be closely related to the phenomenon of lipid peroxidation. The activation of OmpC channels, reconstituted from the outer membrane of *E. coli* in lipid membranes, or of the voltage dependent anion channel, VDAC, from the outer mitochondrial membrane of *N. crassa*, was only observed in membranes of sufficient oxidizability, i.e., not in membranes formed from saturated diphytanoyllecithin [50]. In the case of alamethicin channels, photodynamic activation is suppressed if vitamin E, an antioxidant known to inhibit lipid peroxidation, is added to the membrane (Fig. 9). In this case, only

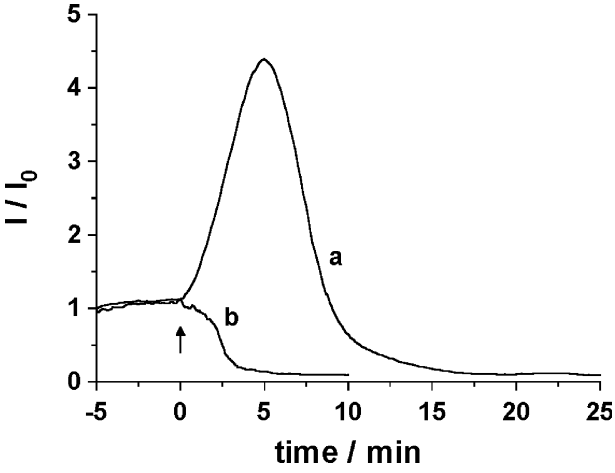

Fig. 9. Photodynamic activation and inactivation of alamethicin channels [50]. The ratio I/I_0 of the current I normalized to the initial current I_0 is plotted as a function of time. The membranes were formed from 1% solutions of dioleoyllecithin in decane containing 0.5 mM rose bengal. The membrane separated two aqueous solutions of 1 M NaCl and 0.25 μM alamethicin. Experiments were performed in the absence (*a*) and presence (*b*) of 2 mg/ml vitamin E in the membrane-forming solution. A constant voltage of 55 mV was applied to the membrane. The initial membrane conductance was about $4 \cdot 10^{-3}$ S/cm^2 in both cases. Illumination was started at time zero (*arrow*). Reproduced from [50] with permission.

inactivation is observed, which in the absence of vitamin E is only visible at longer times of irradiation.

Similar results were obtained for the two K^+ carriers valinomycin and nonactin, which show a pronounced activation behavior if the membrane is exposed to ionizing radiation [100] or to visible light in the presence of the photosensitizer rose bengal [50], but only for lipid membranes of sufficient oxidizability and in the absence of vitamin E. Consequently, activation of the different transport systems seems to be an indirect effect of lipid peroxidation, based on the modification of a physical parameter important for membrane transport.

A physical property, which is known to be of profound influence for ion transport across biological membranes, is the dielectric constant ε_m. This is due to the low value of ε_m (of the order of 2) in the membrane interior as compared with $\varepsilon_a \approx 80$ in the aqueous phases on both sides of the membrane. As a consequence, there is a large electrostatic energy difference for ions between water and membrane interior, which leads to extremely low ion concentrations in the membrane interior [76]. Efficient ion transport across biological membranes is only possible if the positive electrostatic energy difference is (at least partly) compensated by other molecular (hydrophobic) interactions, as in the case of lipophilic ions or ion carriers. The membrane barrier, experienced by the charged species throughout their translocation across the membrane, would be further reduced if the dielectric constant of the membrane is enhanced by lipid peroxidation. This is supported by theoretical and experimental arguments. A simple estimate based on the Born equation (modified for thin lipid structures by Parsegian [76]) shows that a 50% increase of ε_m would lead to an enhancement by almost three orders of magnitude of the translocation rate constant across the membrane [100]. Experimentally, a radiation-induced enhancement of the membrane conductance of (up to) more than two orders of magnitude was observed at carrier-mediated ion transport through lipid membranes [50, 100], and an increase of ε_m up to a factor of two. The modification of ε_m, induced by a photodynamic or a radiolytic treatment of the membrane, was detected by measurement of the membrane capacitance [50, 102].

Ion channels (contrary to ion carriers) provide polar pathways for ions across the hydrophobic membrane barrier. For their proper functioning, the structural element forming the polar pathway must be inserted into the membrane, a process which is considered to be substantially facilitated by an increase of ε_m. As a consequence, the opening rate of active ion channels from an inactive precursor state is expected to be enhanced.

Summarizing the arguments of the last sections, the accumulation of polar products of lipid peroxidation in the membrane interior, via an increase of the dielectric constant of the membrane interior, is thought to give rise to many of the activation phenomena of membrane transport described in the literature.

Inactivation of Membrane Functions by Lipid Peroxidation

Activation of membrane functions is usually followed by inactivation phenomena as the time of exposition to reactive oxygen species is increased. The data in Fig. 9 illustrate an example. Following an initial increase, the conductance induced by alamethicin channels shows a strong decay. The decay is even more pronounced in the presence of the antioxidant vitamin E, which inhibits the initial activation. In the absence of vitamin E, part of the decay is compensated by the initial activation. Inactivation of alamethicin channels, which is not inhibited by vitamin E, seems to be a direct effect of reactive species.

Activation is completely absent at gramicidin channels (*cf.* Figs. 4 and 5) and at polyene channels (Fig. 6). In both cases an eventual activation behavior is buried under the very efficient inactivation phenomena of these very sensitive channel species.

Following the definition given in the Introduction, inactivation of gramicidin A and amphotericin B channels is a direct effect, which has to be distinguished from indirect inactivation induced by lipid peroxidation. Two different phenomena have been envisaged to contribute to the indirect effect:

a) Modification of the lipid environment of a given transport system might deteriorate its functional efficiency.
b) The transport system might be inactivated by secondary reactive species generated throughout the process of lipid peroxidation.

There is a lack of convincing experimental evidence for a). Environmental effects were discussed at carrier-mediated potassium transport at large doses of ionizing radiation [100]. Following a strong initial activation, the electric current carried by valinomycin-potassium complexes shows a decrease (similar to the behavior of alamethicin), which was interpreted via a reduction of the translocation rate constants of the carrier and the carrier-ion complex across the membrane barrier. The reduction was suggested to be the result of cross-linking of lipid radicals leading to an enhanced microviscosity of the membrane at large doses of ionizing radiation [1, 12]. A further possibility is, however, a reduction of the partition coefficient of the hydrophobic ion carriers (i.e., a reduction of the carrier concentration in the membrane) induced by the enhanced polarity of the membrane interior (*see* previous section). Contrary to a), sufficient experimental evidence is available for alternative b), i.e., for inactivation by the action of secondary reactive species. Throughout the peroxidation process and also as end products of this process, a series of new reactive species is generated, which have been found to modify the structure and function of many cellular macromolecules [24, 31–33, 45, 96, 111]. The process of lipid peroxidation is illustrated in Fig. 8. The secondary reactive species include lipid radicals $L^{\cdot}$, peroxylradicals $LOO^{\cdot}$, as well as short chain radicals and reactive aldehydes produced by degradation of lipid hydroperoxides. The cellular constituents, which may be attacked and modified by the secondary reactive species, are not limited to membrane-associated proteins, but also include the genetic substance and even cytoplasmic proteins. This is due to the fact that part of the secondary reactive species are water soluble.

As an example, Fig. 10 shows inactivation of cytoplasmic creatine kinase by adding lipid vesicles, which were exposed to 100 Gy of ionizing radiation before being added to the protein solution. Addition of nonirradiated vesicles had virtually no influence on enzyme activity within the applied time range (*data not shown*). Addition of preirradiated vesicles induces an exponential decay of enzyme activity, which depends on the dose rate applied throughout irradiation of the vesicles. An inverse dose-rate behavior is clearly apparent, i.e., vesicles exposed at smaller dose rates (but to the same total dose) were more efficient. Inverse dose-rate effects were found to be characteristic of lipid peroxidation, as discussed in section 2. Therefore, a greater amount of secondary reactive species is expected, if smaller dose rates are applied. As far as we know, inverse dose rate effects were not observed at radiation-induced protein modifications in the absence of lipids. Therefore, the observation of an inverse dose rate behavior at a protein/lipid mixture may serve as an indicator that the protein modification is an indirect effect caused by lipid peroxidation products. The conclusion is again supported by experiments with creatine kinase [58]. No influence of the dose rate was observed at X-ray-induced inactivation of this enzyme. If, however, lipid vesicles were added to the protein solution, inactivation of the enzyme by irradiation of the mixture clearly showed an inverse dose rate behavior. This is a strong indication that at least part of the inactivation was an indirect effect, i.e., is due to the action of secondary reactive species produced by peroxidation of lipid vesicles. Further details on free radical-induced inactivation of creatine kinase may be found elsewhere [56–58].

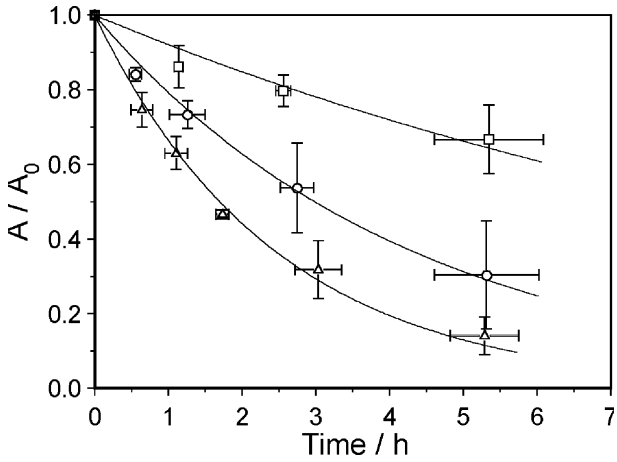

Fig. 10. Inactivation of creatine kinase (MM-CK) by preirradiated vesicles [58]. A suspension of lipid vesicles made from 0.22 mM dilinolenoyllecithin was exposed to 100 Gy at dose rates 98 Gy/min (□), 5.8 Gy/min (O) and 1.6 Gy/min (Δ). Subsequently, 200 µl of the preirradiated vesicle suspension were mixed with 800 µl of MM-CK (62 µg/ml). The mixture was kept at room temperature. Its relative enzyme activity, A/A_0, was followed as a function of time. The data represent mean values (± SD) of 6 different experiments. Horizontal bars account for slightly different times of activity measurements. The characteristic time constant of the exponential inactivation, t_{37}, was calculated as 12.1 h (at 98 Gy/min), 4.3 h (at 5,8 Gy/min), and 2.5 h (at 1.6 Gy/min). Addition of nonirradiated vesicles had no influence on enzyme activity within the applied time range (*data not shown*). Reproduced from reference [58] with permission from Int. J. Rad. Biol., www.tandf.co.uk.

Membrane proteins have a natural lipid environment and are therefore particularly exposed to an eventual attack of secondary reactive species. An inverse dose rate behavior was indeed observed for X-ray-induced inactivation of a series of mitochondrial enzymes and for two important, active ion-transport proteins, the Na,K-ATPase from the plasma membrane of animal cells and the sarcoplasmic Ca-AT-

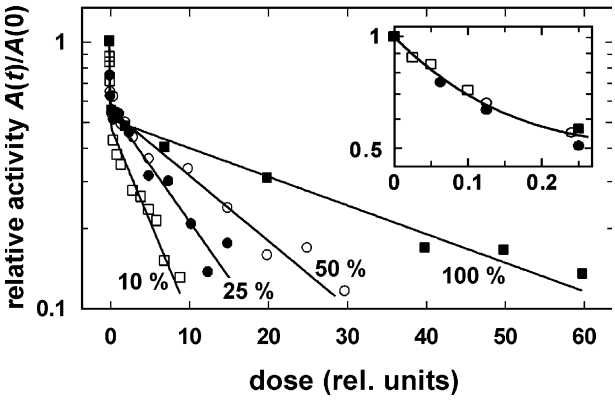

Fig. 11. Photodynamic inactivation of the Na,K-ATPase [51]. The enzyme was prepared from the outer medulla of rabbit kidneys in the form of open membrane fragments and was illuminated by visible light in the presence of the sensitizer rose bengal at different relative light intensities. The relative enzyme activity $A(t)/A(0)$ ($A(0)$ = initial activity before illumination) is plotted as a function of the light dose in arbitrary units, which is proportional to the product of light intensity and duration of illumination. The inset shows the initial, fast decay component at an enhanced resolution. (Reproduced with permission from J. Membrane Biology, reference [51])

Pase, which controls muscle relaxation [40, 58]. In nearly all cases, X-ray-induced inactivation was found to follow a simple exponential decay. A different behavior was observed at the photodynamic inactivation of the Na,K-ATPase and the sarcoplasmic Ca-ATPase. Biexponential inactivation curves were found in these cases [51]. The initial fast decay does not depend on the dose rate, whereas the following slower decay shows an inverse dose rate behavior (*cf.* Fig. 11). Following the arguments mentioned above, the fast decay was explained by a direct photodynamic effect (presumably caused by $^1O_2$) and the slow decay—due to its inverse dose-rate behavior—as an indirect photodynamic phenomenon [51]. In view of their potential importance for the reperfusion damage of the ischemic heart, the oxidative modification of the Na,K-ATPase and of the SR Ca-ATPase has been studied by various groups using different oxidation procedures to simulate the situation in vivo [7, 17, 23, 42, 43, 46, 67, 69, 78, 82, 83, 89, 91, 110, 120]. The direct and the indirect inactivation pathways have been controversially discussed. Fig. 11 provides evidence for a contribution of both.

The application of the dose-rate behavior for distinguishing between direct and indirect inactivation must, however, be supplemented by two restrictive comments:

a) The inverse dose rate behavior at free radical-induced lipid peroxidation is closely associated with the bimolecular radical-radical termination reactions shown in Fig. 8. If termination is primarily due to high concentrations of antioxidants, which act as radical scavengers, the dose rate dependence of the radical chain mechanism disappears. Therefore, indirect inactivation by products of lipid peroxidation cannot be excluded if no dose-rate dependence is observed in an experiment at sufficiently high scavenger concentrations.
b) At photodynamic inactivation, an inverse dose-rate behavior can be induced by oxygen depletion [71, 118]. Therefore, appropriate controls have to be performed to exclude this phenomenon.

Inactivation by products of lipid peroxidation has been found for many protein species and also for the genetic substance [24, 70, 112]. Unfortunately, however, in most cases the dose-rate behavior was not investigated. This also holds for DNA, where the induction of strand-breaks, cross-linking phenomena and base damage were observed.

At the level of organisms (where both, DNA and membrane lipids, are present in each cell), the situation appears contradictory. Russell and Kelly [87] reported a normal dose rate behavior in their classical paper on radiation-induced mutations in mice, i.e., more mutations at higher dose rates. More recent studies showed, however, an inverse dose-rate behavior at sufficiently small dose-rates [19, 68, 81, 119]. An inverse dose-rate behavior of the genetic substance would be of great practical importance for the evaluation of the genetic risk and of the cancer risk associated with the use of ionizing radiation. Current estimates are largely based on studies of the survivors of the atomic bombings of Hiroshima and Nagasaki, i.e., from a radiation exposure at a very high dose rate. Extrapolation to low dose rates might underestimate the risks if the induction of mutations exhibits an inverse dose-rate behavior.

Consequences of Oxidative Membrane Damage for the Membrane Potential of the Plasma Membrane

So far membrane damage has been considered at the level of individual proteins such as ion channels and ion pumps. The last section of this review will be devoted to membrane potentials, which are of profound influence for many physiological phenomena. The depolarization of the plasma membrane is an early membrane modification, which precedes the inactivation of transport pathways for amino acids or sugars or more serious membrane damage such as the disruption of the membrane barrier for large molecules [52, 94]. It is well known that membrane potentials are based on three fundamental conditions: the existence of a) active ion transport systems (ion pumps), and b) ion-selective, passive transport

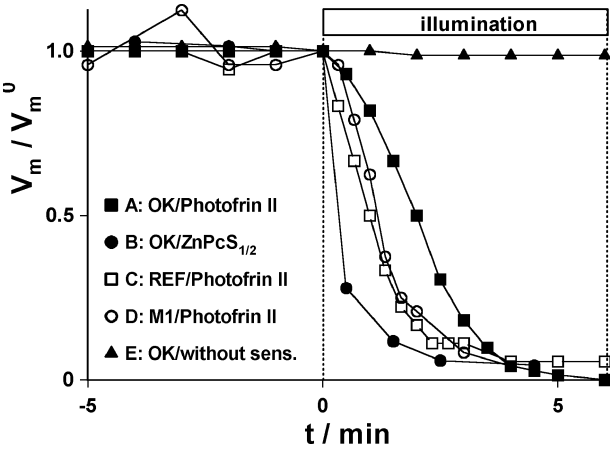

Fig. 12. The decay of the membrane potential following illumination of different cell types in the presence of either photofrin II or Zn-phthalocyanine. The potential was detected using the whole-cell configuration of the patch-clamp technique (*see* [62] for details). *OK* = opossum kidney, *REF* = rat embryo fibroblast, *M1* = REF immortalized by transfection with the c-myc oncogen. The resting potential V_m^0 before illumination was −77 mV (OK-cells), −22 mV (REF-cells) and −28 mV (M1-cells). Reproduced from reference [62] with permission from J. Membrane Biology.

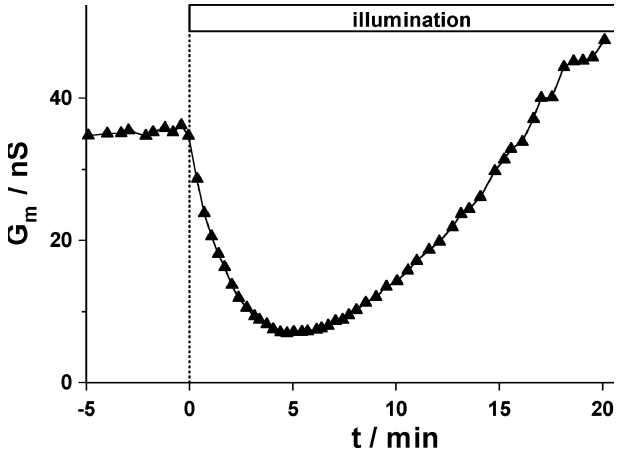

Fig. 13. Time dependence of the membrane conductance, G_m, of OK-cells throughout illumination in the presence of the sensitizer photofrin II. The experiment was performed using the whole-cell configuration of the patch-clamp technique and in the presence of high concentrations of KCl (about 150 mM) on both sides of the membrane [62]. Reproduced with permission, from reference [62].

pathways (ion channels). c) A further important requirement for the existence of membrane potentials are the excellent barrier properties of biological membranes. Passive ion fluxes through biological membranes are essentially confined to ion channels, i.e., leak currents are minimized.

All three requirements are negatively affected by an oxidative modification of the membrane. The activity of ion pumps is reduced (*see* previous section), ion channels become inactivated and leak currents are strongly increased, as was shown for photosensitized membrane damage by Valenzeno and Tarr [105–108, 113–116] and by our group [49, 61–64] by using whole-cell and single-channel configurations of the patch-clamp technique.

Figure 12 illustrates the behavior of the membrane potential, V_m, for three different cell types and two different photosensitizers [62]. Throughout illumination of the cells, V_m is reduced to virtually zero within several minutes. The same behavior was found for other membrane-active photosensitizers [49]. The decay of V_m is induced by an initial inactivation of potassium channels and by a subsequent strong increase of the leak conductance of the membrane. The two phenomena are clearly apparent from the behavior of the whole-cell membrane conductance. G_m (Fig. 13), and from an analysis of membrane patches (Figs. 14 and 15). The conductance decay in Fig. 13 was only observed in the presence of K^+. Direct evidence for the inactivation of K^+ channels was obtained by single-channel measurements. Following an initial activation (*see* also sec-

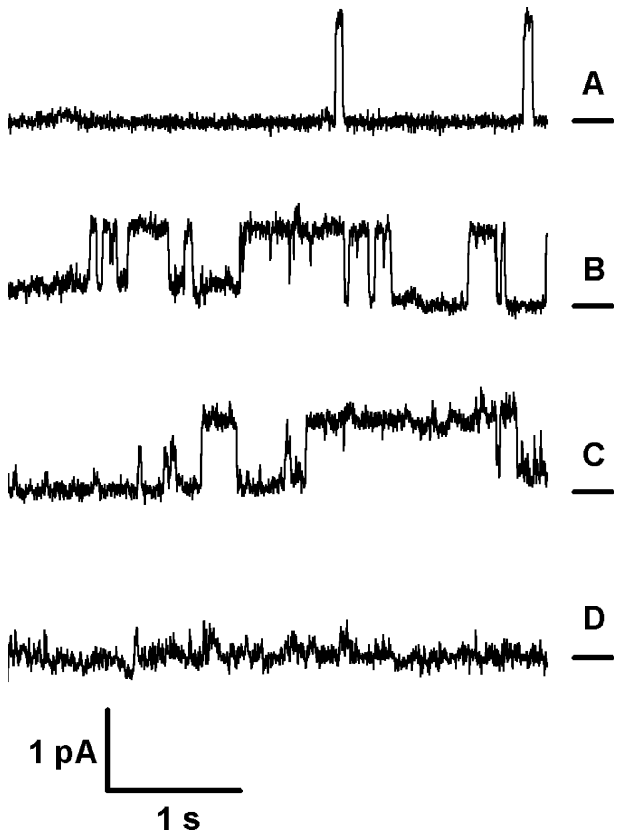

Fig. 14. Photodynamic activation followed by inactivation of the sK-channel of the OK plasma membrane induced by illumination in the presence of photofrin II. Traces of the membrane current at a constant holding potential of V_{hold} = 20 mV before (*A*), 138 s after (*B*), 154 s after (*C*) and 228 s after (*D*) start of illumination. The bars on the right-hand side indicate the current level in the absence of open channels (*see* [63] for further experimental details). Reproduced from reference [62] with permission.

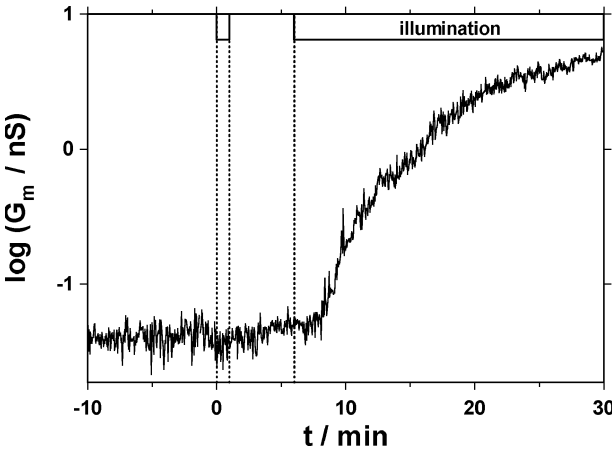

Fig. 15. Photodynamically induced leak conductance of a membrane patch (obtained from an OK-cell) by illumination in the presence of photofrin II. The induction of the conductance increase requires a sufficiently long illumination period (*see* [63] for details). Reproduced from reference [63]. with permission.

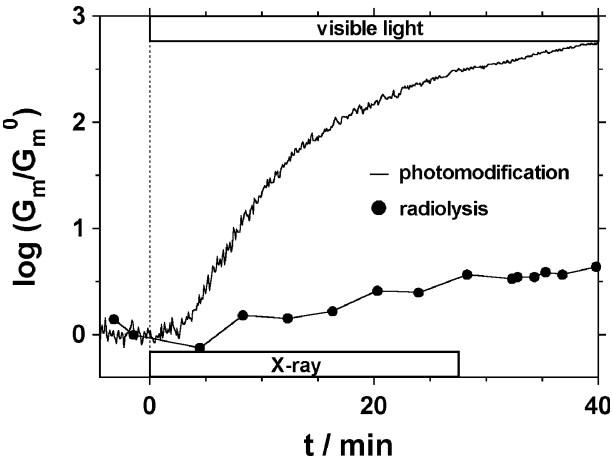

Fig. 16. Comparison of the time dependence of the conductance, G_m, of a membrane patch in the absence of ion channels in order to study the modification of the leak conductance of the membrane throughout oxidative damage. The patch was either exposed to 80 kV X-rays (filled circles) or to visible light in the presence of photofrin II (continuous line). The total X-ray dose applied was 650 Gy with a dose rate of 25 Gy/min. The conductance, G_m^0, before irradiation ignore was in the range 100–200 pS (*see* [64] for further experimental details). Reproduced from reference [64] with permission from Int. J. Rad. Biol., www.tandf.co.uk.

tion Activation of Membrane Transport), inactivation was observed for a small-conductance K^+ channel (sK, *see* Fig. 14), for a stretch-activated nonselective potassium channel (SA-cat) and for the large-conductance, Ca^{2+}-dependent potassium channel (maxi-K_{Ca}) [63]. The increase of the unspecific leak conductance of the plasma membrane was deduced from the pronounced increase of the membrane conductance observed after about 5 minutes of illumination (Fig. 14) and was supported by the investigation of membrane patches in the absence of ion channels [61, 63]. A strong conductance increase by at least three orders of magnitude was observed when the membrane patch was illuminated in the presence of photofrin II (Fig. 15) or of other membrane-active photosensitizers [49].

The data shown in Figs. 13–15 were all obtained from opossum kidney cells. Qualitatively similar results were, however, found in electrophysiological studies of membrane photomodification of other cell types, in earlier studies of the squid axon [75] and the lobster axon [79, 80], and in the more recent studies of cardiac myocytes from frog [105–108, 114, 115]. Most of these studies were performed in order to understand the influence of photosensitizer-generated reactive species on the action potential.

The strong increase of the leak current, apart from its contribution to the depolarization of the membrane potential, seems to be of relevance for photosensitized cell killing. We found that the enhanced leak conductance of the plasma membrane is rather unspecific and also allows permeation of Ca^{2+} ions from the extracellular medium [63]. The resulting increase of the cytoplasmic Ca^{2+} concentration could function as a trigger for further intracellular processes, finally leading to photosensitized cell death [5, 109, 116, 117].

The increase of the leak conductance shown in Figs. 13 and 15 is followed by a breakdown of the bilayer structure if oxidative damage is continued. This may be concluded from the strong decrease of the stability of planar lipid membranes. The corresponding investigations have been summarized in a previous review [98].

To conclude this chapter, a comparison between the photodynamic method and the method of water radiolysis, the two procedures that have mainly been applied to study oxidative membrane damage throughout the present review, will be performed on the same system. The most reactive species generated by water radiolysis is the hydroxyl radical, while photodynamic effects are largely due to the action of singlet oxygen. The oxidizing potential of $OH^{\cdot}$ is certainly larger than that of $^1O_2$. Oxidative membrane damage depends, however, on the concentration of the reactive species inside the membrane. The hydroxyl radical is generated in water. There is a high probability that it will react with a constituent of the aqueous phase before it arrives at the membrane/water interface. The formation of singlet oxygen depends on the location of the sensitizer molecules. In the case of membrane-active sensitizers, $^1O_2$ will be formed directly inside the membrane. Keeping this in mind, it does not come as a surprise when considering the result shown in Fig. 16, namely, a considerably larger effect of visible light in the presence of photosensitizers as compared with the effect of large doses of ionizing radiation. This holds not only for the increase of the leak conductance of the membrane but

also for the depolarization of the membrane potential [64].

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