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Photofrin II Sensitized Modifications of Ion Transport Across the Plasma Membrane of an Epithelial Cell Line: II. Analysis at the Level of Membrane Patches

L. Kunz, G. Stark
Department of Biology, University of Konstanz, Box M638, D-78457 Konstanz, Germany

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Abstract. In the first part of this study, photofrin II sensitized membrane modifications of OK-cells were investigated at the level of macroscopic membrane currents. In this second part, the inside-out configuration of the patch-clamp technique is applied to analyze the phenomena at the microscopic level. It is shown that the characteristic single channel fluctuations of the electric current disappear after the start of illumination of membrane patches in the presence of photofrin II. This holds for all three types of ion channels investigated: the large-conductance Ca^{2+}-dependent K^+ channel (maxi-K_{Ca}), a K^+ channel of small conductance (sK), and a stretch-activated nonselective cation channel (SA-cat). Part of the experiments show a transient activation of the channels (indicated by an increase of the probability in the open-channel state) before the channels are converted into a closed nonconductive state. Inactivation of all three channel types proceeds by a continuous reduction of their open probability, while the single channel conductance values are not affected. The process of photodynamically induced channel inactivation is followed by a pronounced increase of the leak conductance of the plasma membrane. The latter process — after light-induced initiation — is found to continue in the dark. The ionic pathways underlying the leak conductance also allow permeation of Ca^{2+} ions. The resulting Ca^{2+}-flux may contribute to the photodynamically induced increase of the intracellular Ca^{2+} concentration observed in various cell lines.

Key words: Photosensitization — Photofrin II — Patch-clamp — OK-cells — Ion channels — Leak conductance

Correspondence to: G. Stark

Introduction

The influence of photosensitized membrane damage on ion transport across biological membranes has so far been investigated via the modification of the membrane potential and via the inactivation of macroscopic electrical currents (Pooler, 1987; Valenzeno, 1987; Specht & Rodgers, 1990; Tarr & Valenzeno, 1991; Valenzeno & Tarr, 1995; Kunz & Stark, 1998). The use of the patch-clamp technique in principle allows a more detailed analysis of the underlying molecular events, e.g., by studying the functional modification of individual cellular ion channels during a photodynamic treatment of the membrane. Studies of this kind, which require application of the inside-out configuration of this technique have, however, been complicated so far by the strong current fluctuations accompanying the almost simultaneous increase of the leak conductance of the membrane (Valenzeno & Tarr, 1995). Using opossum kidney (OK) cells as model system and photofrin II as sensitizer, we have recently been able to separate the two phenomena and to show photodynamically induced inactivation of single ion channels of two different types (Kunz & Stark, 1997). Our previous brief report is now supplemented by a more detailed study of the behavior of three different kinds of ion channels and by a correlation of the macroscopic (whole-cell) membrane currents (described in part I of this series) with the microscopic properties of the currents analyzed at the level of membrane patches. A correlation between the currents of single- and multiple-channel systems has so far only been possible in the case of the model channels formed by gramicidin A and amphotericin B in lipid membranes (Kunz et al., 1995; Koufen, Zeidler & Stark, 1997). In addition the paper provides electrophysiological evidence for an enhanced Ca^{2+} permeability of the photodynamically induced leak conductance of the plasma membrane. This could be of

consequence for the initiation of the intracellular signaling cascade finally leading to photosensitized cell death (Ben-Hur & Dubbelman, 1993).

Materials and Methods

The experiments of part II of this study were performed by application of the inside-out configuration of the patch-clamp technique (Hamill et al., 1981). Most of the experimental conditions agreed with those mentioned in part I (Kunz & Stark, 1998). The solutions applied throughout the experiments were a high-NaCl solution (140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$) and a high-KCl solution (135 mM KCl, 20 mM NaCl). The concentration of free Ca^{2+} of the high-KCl solution was varied by addition of different combinations of concentrations of $CaCl_2$ and EGTA (in brackets): 0.1 μM free Ca^{2+} (0.49 mM $CaCl_2$, 0.99 mM EGTA), 1 μM free Ca^{2+} (0.98 mM $CaCl_2$, 1.09 mM EGTA) or 1 mM free Ca^{2+} (1 mM $CaCl_2$). Additionally, the solutions contained 1 mM $MgCl_2$, 10 (or 20) mM HEPES and 10 (or 18) mM glucose. The pH was adjusted to 7.4 by adding 1M NaOH or 1M KOH.

The heat-polished borosilicate patch pipettes filled with pipette solution had an input resistance of about 4–12 MΩ. The current traces were low pass filtered at 1.3 kHz and sampled with a rate of 4.0 kHz. Evaluation of the probabilities in the open channel state was performed using standard procedures. For details as well as for a more elaborated description of the experiments *see* Kunz (1998).

The ion selectivity of the enhanced leak conductance after a photodynamic treatment was estimated via zero-current potential measurements as follows. Inside-out patches were established with the high-NaCl solution in bath and pipette. The bath solution was exchanged by one of the solutions in Table I. The membrane patch was then illuminated until the resistance was reduced to a value of less than 1 GΩ. The zero-current potential, $V_{z,1}$, was measured in the current-clamp mode. After disruption of the patch, the potential was measured again ($V'_{z,2}$). After correction of $V'_{z,2}$ for the diffusion potential, V_d, of the pipette, $V_{z,2}$ is obtained. The difference $\Delta V_z = V_{z,1} - V_{z,2}$ was used to estimate relative permeabilities for different ions (*see* Appendix and Table 2). By way of this method, the offset potential, V_{offset}, is eliminated. The latter is of the order of a few mV and is produced by the diffusion potentials at the ends of the agar bridge (filled with 150 mM KCl) between the two aqueous bath solutions of reference electrode and patch pipette, by asymmetries of the electrodes and by the amplifier. V_d was calculated from the Henderson-Planck Equation (using a computer program obtained from Dr. F. Mendez, Göttingen).

Results

Photodynamic effects were studied at three types of ion channels: the large-conductance Ca^{2+}-dependent K^+ channel (maxi-K_{Ca}), the K^+ channel of small conductance (sK) and the mechanosensitive (or stretch-activated) nonselective cation channels (SA-cat). These three types of channels in OK-cells have been characterized by different authors (Ubl, Murer & Kolb, 1988; Kolb, 1990; Hollunder-Reese et al., 1991; Ubl, 1992) and can be distinguished via their single channel conductance, their zero-current potential at asymmetrical distribution of ion concentrations, their Ca^{2+}-dependence and via their characteristic fluctuation pattern.

The main characteristics of the maxi-K_{Ca} are its large mean single channel conductance ($\overline{\Lambda} = 202 \pm 25$ pS, $n = 27$, under the experimental conditions of Fig. 1) and its strong activation with increasing Ca^{2+} concentration. The magnitude of the single channel conductance of SA-cat and sK-channels is tenfold lower (about 20 pS, and 18 ± 4 pS, $n = 12$, respectively, under the experimental conditions of Figs. 2 and 3). The channels may be distinguished from one another via their different cation selectivity and fluctuation behavior. Figs. 1–3 illustrate the single channel fluctuations of the three channel types before and during illumination in the presence of photofrin II. The main differences in the normal fluctuation pattern are as follows. The maxi-K_{Ca}, at sufficiently high Ca^{2+} concentrations, is mainly in the open channel state and shows brief flickerings to the closed state (Fig. 1*a*). Fluctuations induced by stretch-activated (SA-cat) channels usually indicate the simultaneous presence of more than one channel (*see* Fig. 2*a*). A characteristic feature of the sK-channel is its comparatively short dwell time in the open state (Fig. 3*a*).

In all three cases a similar behavior is observed after the start of illumination of the inside-out patch. The disappearance of the fluctuations indicates a continuous reduction of the probability in the open-channel state. The inactivation of the channels clearly precedes the characteristic changes of the basic membrane conductance. Traces D in Figs. 1*a* and 3*a* only indicate the beginning of a strong increase of the noise amplitude and of the mean current, which — after a comparatively short period of the time of illumination — prevent the detection of the typical single channel fluctuation pattern (*see below*). Therefore, the light-induced transition into a closed channel state may be analyzed only within a short time window. In the case of OK-cells, the time window is, however, long enough to observe the decay of the channel activity for all three channel types investigated.

In some cases, prior to inactivation, a transient activation of the channels was observed ($n = 5$), which, in the case of the sK-channel, is illustrated in Fig. 4. Activation is detected via an increased frequency of transitions from the closed into the open-channel state (Fig. 4*a*). At the same time, the dwell time in the open state is increased by a factor of 5 (Fig. 4*c*). As a consequence, a transient increase of the mean probability, P_o, in the open state is obtained (Fig. 4*b*). A similar behavior has also been found for the two other channel types. Activation could, however, be resolved only when the inactivation process was comparatively slow. At the inactivation of the maxi-K_{Ca}, a partial recovery of the channel activity was observed after the end of illumination in some of the experiments ($n = 4$, *data not shown*).

Activation and inactivation of ion channels is followed by additional strong changes of the conduction properties of the plasma membrane. This is concluded from the increase of the current noise (*see* traces D in

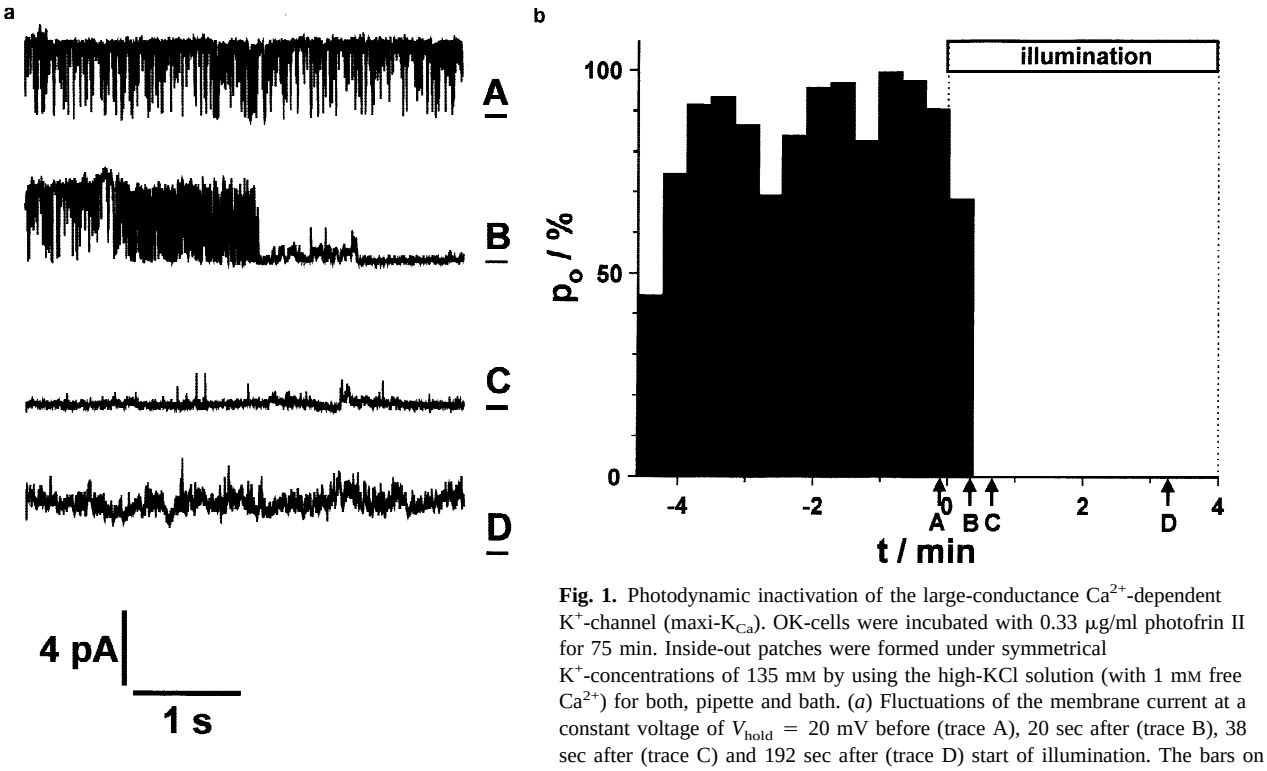

Fig. 1. Photodynamic inactivation of the large-conductance Ca^{2+}-dependent K^+-channel (maxi-K_{Ca}). OK-cells were incubated with 0.33 μg/ml photofrin II for 75 min. Inside-out patches were formed under symmetrical K^+-concentrations of 135 mM by using the high-KCl solution (with 1 mM free Ca^{2+}) for both, pipette and bath. (*a*) Fluctuations of the membrane current at a constant voltage of $V_{hold} = 20$ mV before (trace A), 20 sec after (trace B), 38 sec after (trace C) and 192 sec after (trace D) start of illumination. The bars on the right hand side indicate the current level in the absence of open channels. Data were additionally filtered with 500 Hz. The single channel conductance of the channel was 190 pS. (*b*) The reduction of the open probability, P_o, after illumination of the membrane. P_o was calculated by averaging over subsequent time intervals of 20 sec. The letters A, B, C and D indicate the time of measurement of the current traces shown in *a*.

Figs. 1*a* and 3*a*), the appearance of burstlike irregular fluctuations (*not shown*) and a strong increase of the basic (leak) conductance of up to three orders of magnitude at least (Figs. 5 and 6). All effects are only observed after illumination in the presence of the sensitizer. The increase of the leak conductance requires a minimum time of illumination. The conductance, G_m, is not affected by the first short illumination period (Fig. 5), and shows a strong increase only during the following continuous illumination. It is throughout the time interval of the unmodified (original) leak conductance that the inactivation of ion channels may be observed (*see above*). The second experiment (Fig. 6) indicates that continuous illumination is not required to obtain a strong increase of the leak conductance. The conductance increase starts after an illumination period of adequate length and continues in the dark. This shows that a light-induced initiation process is sufficient for the further progress of the conductance increase. The phenomenon is believed to represent a structural modification of the lipid component of the plasma membrane (*see* Discussion).

The strong increase of the leak conductance of the membrane may be applied to generate a new, light-induced slow-whole-cell version (perforated patch) of the patch-clamp technique. Illumination of the cell in the cell-attached configuration (with photofrin II or $ZnPcS_{1/2}$ present in the pipette solution only) allows an increase in the leak conductance of the patch in such a way that the properties of the whole cell, such as the resting potential or the current-voltage relationship may be detected (*see* part I of this series). The sensitizer is only present in the patch under these conditions, so that the conductance of the rest of the plasma membrane is not affected by the photodynamic treatment. The method represents an alternative to the addition of the channel formers nystatin (Horn & Marty, 1988), amphotericin B (Rae et al., 1991) or gramidicin A (Ebihara et al., 1995; Kyrozis & Reichling, 1995); the usual methods to lower the resistance of the membrane patch in order to obtain a perforated patch. The detection of the whole-cell properties in the cell-attached configuration also indicates that the seal resistance of the patch is not affected by the photodynamic treatment. This supports our interpretation that the photodynamically induced increase of the conductance, which is observed throughout an experiment, is indeed a modification of the plasma membrane and is not an artifact due to a deterioration of the seal (*see* part I).

The leak current shows no preference of K^+ over Na^+, as was shown in part I of this study (Kunz & Stark, 1998). The question arises, whether the leak conductance discriminates between cations and anions. A fur-

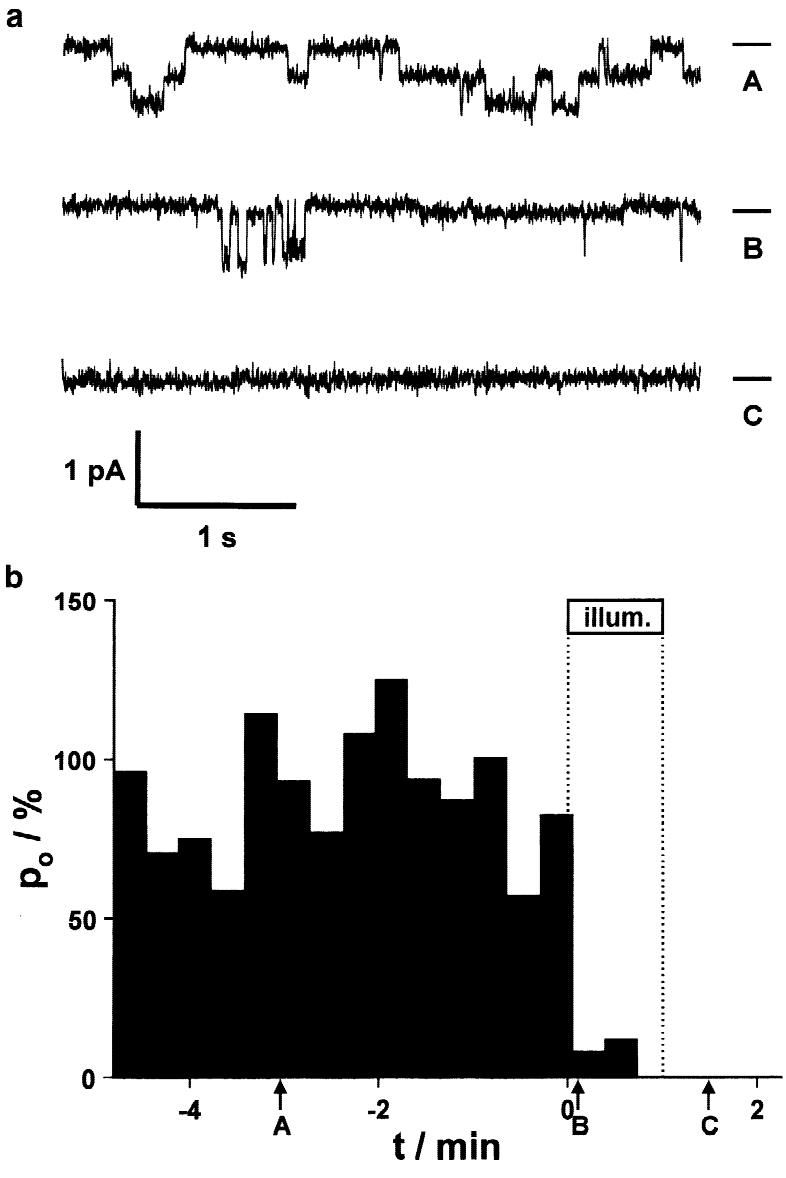

Fig. 2. Photodynamic inactivation of the stretch-activated nonselective cation channel (SA-cat). OK-cells were incubated with 0.33 μg/ml photofrin II for 60 min. Inside-out patches were formed under symmetrical K^+-concentrations of 135 mM by using the high-KCl solution for both, pipette and bath. (*a*) Fluctuations of the membrane current at a constant voltage of $V_{hold} = -20$ mV before (trace A), 6 sec after (trace B) and 89 sec after (trace C) start of illumination. The bars on the right hand side indicate the current level in the absence of open channels. Data were additionally filtered with 250 Hz. The mean single channel conductance was 20 pS, the mean zero-current potential −3 mV. (*b*) The reduction of the open probability, P_o, after illumination of the membrane. P_o was calculated by averaging over subsequent time intervals of 20 sec ($P_o = 200\%$, if both fluctuating channels are open). The letters A, B, and C indicate the times of measurement of the current traces shown in *a*.

ther important issue deals with the Ca^{2+} permeability of the modified plasma membrane. The ion selectivity of biological membranes may be investigated in principle by measurement of zero-current potentials in the presence of asymmetrical distributions of ion concentrations. The method, however, cannot be applied to inside-out patches under normal physiological conditions. As a consequence of the very high resistance of the inside-out patch (in the range of tens of Gigaohms), erroneous potentials produced by the electronic circuit are often observed. The technical problems are not present if the membrane resistance is reduced to a value $\leqslant 1$ GΩ by photodynamic treatment. Thus the measurement of zero-current potentials may be used to study the ion selectivity of the enhanced leak conductance. Measurements were performed, when the conductance of the patch was larger than 10^{-9} S (i.e., the resistance smaller than 1 GΩ). In this case the membrane conductance is virtually completely determined by the new ionic pathways through the membrane so that a further increase of the conductance (i.e., a further increase of the number of the new pathways) has no influence on the zero current potential from which the ion selectivity was derived.

The results obtained for monovalent cations and anions are summarized in Table 2. To estimate the relative permeabilities of Na^+ and Cl^-, the membrane potential was studied with the high-NaCl solution (*cf.* Materials and Methods) in the pipette and with the bath solutions Na 1 or Na 2 of smaller NaCl concentrations (*cf.* Table 1). The other bath solutions shown in Tables 1 and 2 were used to estimate the permeabilities of the choline- and tetraethylammonium cations, and of the glutamate

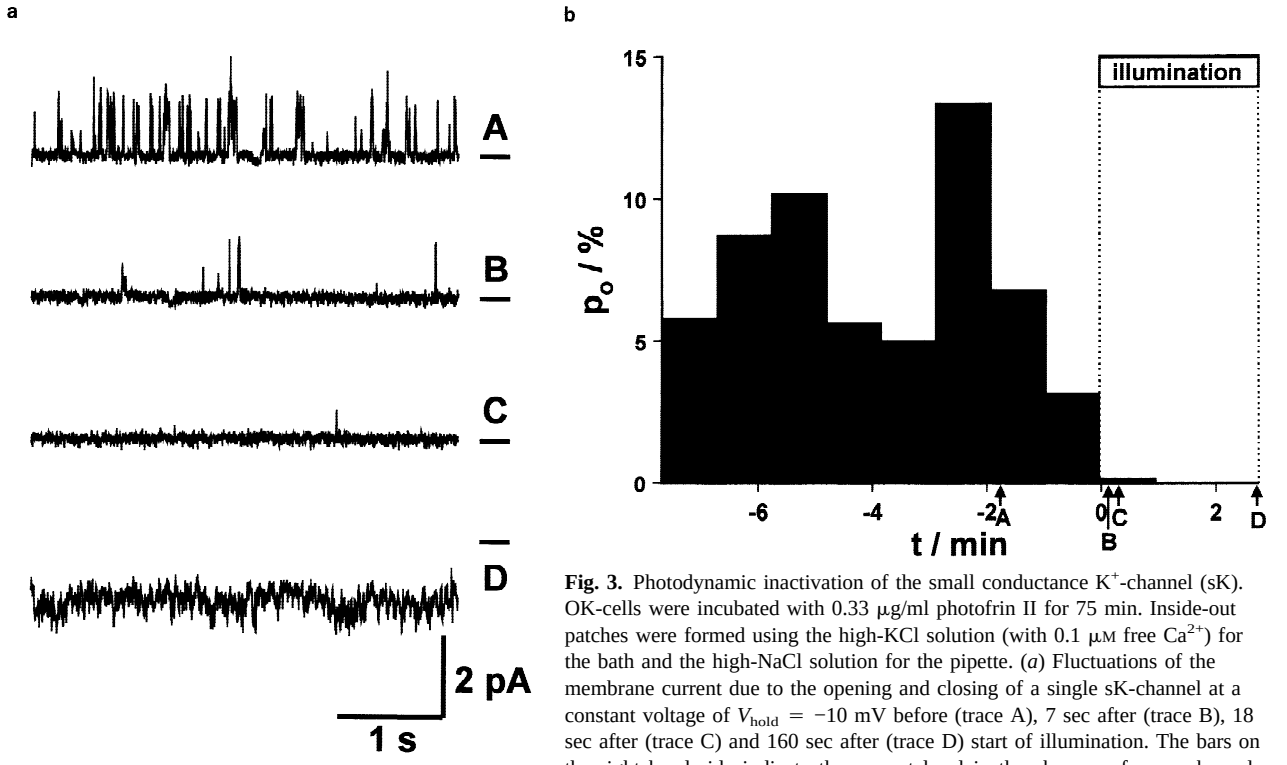

Fig. 3. Photodynamic inactivation of the small conductance K^+-channel (sK). OK-cells were incubated with 0.33 μg/ml photofrin II for 75 min. Inside-out patches were formed using the high-KCl solution (with 0.1 μM free Ca^{2+}) for the bath and the high-NaCl solution for the pipette. (*a*) Fluctuations of the membrane current due to the opening and closing of a single sK-channel at a constant voltage of $V_{hold} = -10$ mV before (trace A), 7 sec after (trace B), 18 sec after (trace C) and 160 sec after (trace D) start of illumination. The bars on the right hand side indicate the current level in the absence of open channels. Data were additionally filtered with 300 Hz. The single channel conductance of the channel was 26 pS, the zero-current potential −64 mV under the conditions applied. (*b*) The reduction of the open probability, P_o, after illumination of the membrane. P_o was calculated by averaging over subsequent time intervals of 60 sec. The letters A, B, and indicate the times of measurement of the current traces shown in *a.*

anion relative to Cl^-. The applied procedure is based on the validity of the Goldman-Hodgkin-Katz Equation and its underlying simplifying assumptions, as is outlined in the Appendix in more detail. The values should, therefore, be considered as a rough estimate only. The data indicate a considerable permeability also for the larger cations and anions.

The same procedure was applied to investigate the Ca^{2+} permeability of inside-out patches of the photomodified plasma membrane. The normal Goldman-Hodgkin-Katz Equation only considers monovalent cations and anions. A procedure based on a similar kind of approach (*see* Appendix) allows, however, the derivation of the relative permeability, $P'_{Ca} = P_{Ca}/P_{Na}$, of Ca^{2+} *vs.* Na^+ from Eq. (1), if the relative permeability, $P'_{Cl} = P_{Cl}/P_{Na}$, of Cl^- to Na^+ is known:

$$P'_{Ca} = (A - Be^u) \cdot (1 + e^u)/(4([Ca^{2+}]_{pip}\, e^{2u} - [Ca^{2+}]_{bath})), \text{ with } A = [Na^+]_{bath} + P'_{Cl} \cdot [Cl^-]_{pip} \text{ and } B = [Na^+]_{pip} + P'_{Cl} \cdot [Cl^-]_{bath}. \quad (1)$$

$u = V_m F/RT$ is the reduced membrane potential difference determined experimentally (R = gas constant, T = temperature in Kelvin, F = Faraday constant). V_m corresponds to the zero-current potential difference, ΔV_z, described in the section Materials and Methods.

Table 3 summarizes data from an experiment with a high NaCl concentration in the pipette, a high $CaCl_2$ concentration in the bath solution (at almost identical Cl^- concentrations) and an experimentally determined value of $V_m = -3{,}6$ mV (i.e., $u = -0.14$). If $P_{Ca} = 0$, the Goldman-Hodgkin-Katz Equation for monovalent ions may be used to estimate V_m (with P'_{Cl} calculated from Table 2). At the concentrations applied, $V_m = \psi_{pip} - \psi_{bath} = -25.5$ mV is obtained in this case, a value far more negative than the experimental value mentioned above. The difference is a qualitative argument for a Ca^{2+} current into the pipette, i.e., for $P_{ca} > 0$. Application of Eq. (1) yields similar values for P_{Ca} and P_{Na} ($P'_{Ca} = 0.8$). This again is a rough estimate only, in view of the assumptions introduced to derive Eq. (1). The applied procedure provides, however, at least qualitative evidence for a considerable permeability of the photomodified plasma membrane for divalent cations such as Ca^{2+}.

Discussion

Illumination of inside-out patches of OK-cells in the presence of photofrin II was found to induce at least two different kinds of processes, activation and inactivation

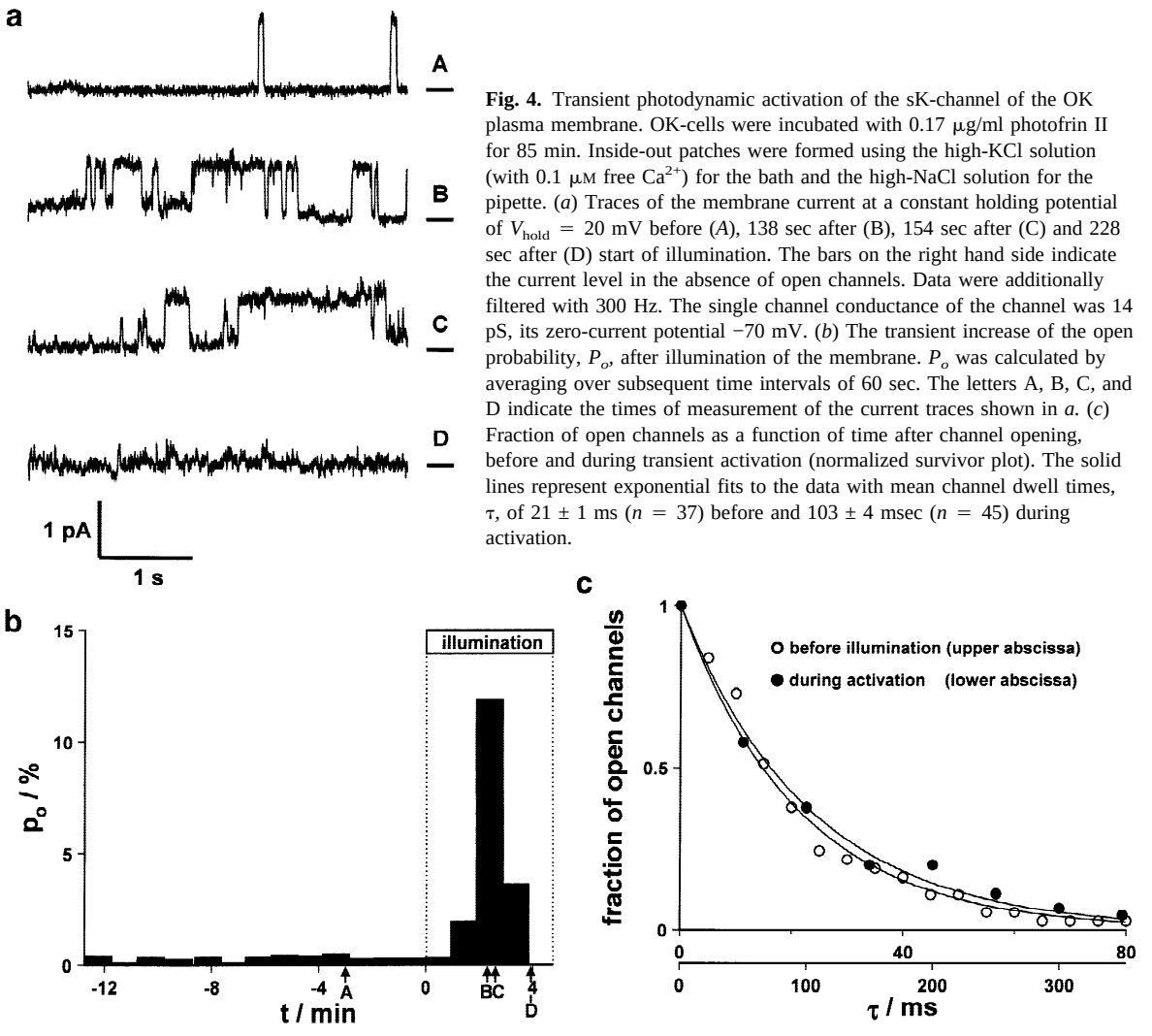

Fig. 4. Transient photodynamic activation of the sK-channel of the OK plasma membrane. OK-cells were incubated with 0.17 μg/ml photofrin II for 85 min. Inside-out patches were formed using the high-KCl solution (with 0.1 μM free Ca^{2+}) for the bath and the high-NaCl solution for the pipette. (*a*) Traces of the membrane current at a constant holding potential of $V_{hold} = 20$ mV before (*A*), 138 sec after (B), 154 sec after (C) and 228 sec after (D) start of illumination. The bars on the right hand side indicate the current level in the absence of open channels. Data were additionally filtered with 300 Hz. The single channel conductance of the channel was 14 pS, its zero-current potential −70 mV. (*b*) The transient increase of the open probability, P_o, after illumination of the membrane. P_o was calculated by averaging over subsequent time intervals of 60 sec. The letters A, B, C, and D indicate the times of measurement of the current traces shown in *a*. (*c*) Fraction of open channels as a function of time after channel opening, before and during transient activation (normalized survivor plot). The solid lines represent exponential fits to the data with mean channel dwell times, τ, of 21 ± 1 ms ($n = 37$) before and 103 ± 4 msec ($n = 45$) during activation.

of ion channels, and an increase of the leak conductance of the membrane. Light-induced modification of ion channels seems to represent a rather general phenomenon, since similar results were obtained for all three types of channels investigated. Following the start of illumination, there is a transient, brief period of activation, i.e., a period of an increased probability, P_o, in the open channel state (Fig. 4). It is followed by the inactivation of the channels, indicated by the disappearance of the typical single channel fluctuations (Figs. 1–3). The largely identical behavior of the three channel proteins suggests a common mechanistic basis. In principle, two alternative ways of modification may be envisaged. The functional changes observed might be caused by a photomodification of sensitive amino acids of the channel proteins, as in the case of the artificial channel formed by the peptide gramicidin A (Sträßle & Stark, 1992; Rokitskaya et al., 1993, 1996; Kunz et al., 1995). Photodynamically induced oxidation and fragmentation at the four tryptophan residues of the peptide were observed in this case (Kunz et al., 1995). Alternatively, the functional changes of the channels might be caused indirectly, namely via a modification of the lipid environment of the channel proteins. Photoperoxidation of the membrane lipids (Girotti, 1990) may be imagined to influence the function of membrane proteins in two different ways, via a change of the physical properties of the bimolecular lipid matrix and via an interaction of the proteins with reactive species generated throughout the lipid peroxidation process.

There is no definite conclusion on the kind of mechanism at present. The similar behavior of all three types of ion channels rather supports the second alternative, i.e., photodynamically induced lipid peroxidation, as the common basis of the functional modifications of the channel proteins. This hypothesis is in line with various studies of other authors, who reported on activation and inactivation processes of ion channels after different oxidative modifications of biological membranes. Phenomena similar to those in the present communication

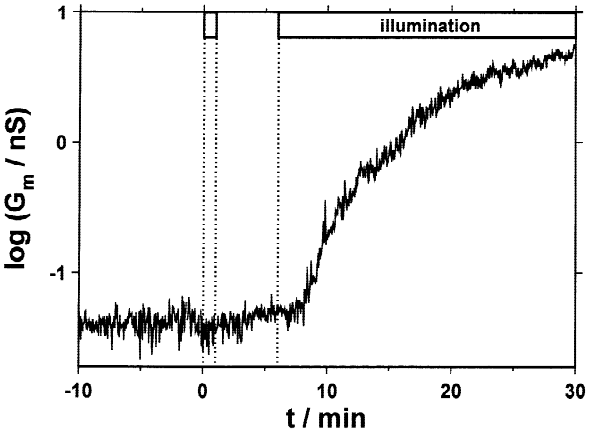

Fig. 5. The photodynamically induced leak conductance, G_m. The induction of the conductance increase requires a sufficiently long illumination period. OK-cells were incubated with 0.33 μg/ml of the sensitizer for 60 min. Inside-out measurements were performed under symmetrical conditions using the high-NaCl solution for both, pipette and bath.

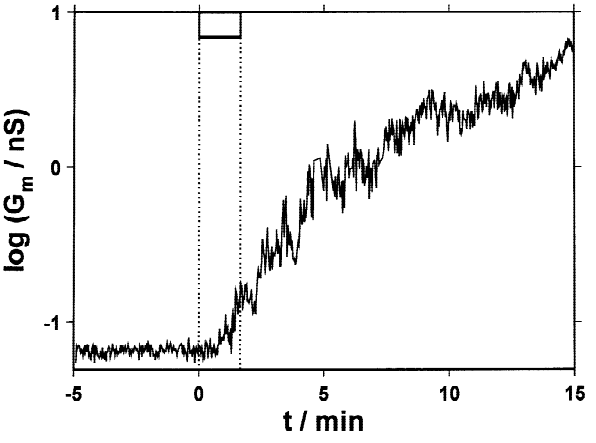

Fig. 6. The photodynamically induced leak conductance, G_m. The conductance increase continues after the illumination period. OK-cells were incubated with 0.83 μg/ml of the sensitizer for 120 min. Inside-out measurements were performed under symmetrical conditions using the high-NaCl solution for both, pipette and bath.

have been found for the Ca^{2+}-release channel from sarcoplasmic reticulum of skeletal muscle (Holmberg et al., 1991; Xiong et al., 1992; Favero, Zable & Abramson, 1995) and for a nonselective cation channel in calf vascular endothelial cells (Koliwad, Kunz & Elliott, 1996). There is extensive literature on the regulation of ion channels by arachidonic acid and its metabolites as well as by other fatty acids (Meves, 1994; Petrou et al., 1995; Baron, Frieden & Bény, 1997; Twitchell, Peña & Rane, 1997). These latter studies show that constituents of the lipid phase as well as their degradation products and metabolites may have a profound influence on the functional properties of ion channels. We think that this

Table 1. Bath solutions for the selectivity measurements

Solution	[NaCl]/mM	
Na 1	10	
Na 2	80	
TEA	5	139 mM TEA-Cl
Glutamate		150 mM Na-glutamate
Choline	5	140 mM choline-Cl

Additionally, the solutions contained 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES and 18 mM glucose. The pH value of all solutions was adjusted to 7.4 with 1M NaOH. This led to an increase of the Na^+ concentration (*see* Table 2 for final concentrations). The solutions Na 1 and Na 2 contained 280 and 140 mM sucrose, respectively, to obtain an osmolarity of about 320 mOsm. (TEA = tetraethylammonium)

could also be valid for products of lipid peroxidation, which have been directly identified by ESR-techniques (Buettner, Kelley & Burns, 1993; Kelley, Buettner & Burns, 1997) and by HPLC-analysis (Valenzeno & Tarr, 1995) after a photodynamic treatment.

Lipid peroxidation might also be responsible for the strong modification of the leak conductance of the plasma membrane. This is supported by the finding of similar effects at artificial lipid membranes, i.e., in the absence of membrane proteins (Mirsky, Stozhkova & Szitó, 1991). The term leak conductance is used to describe the basic conductance properties of the membrane in the absence of the typical current fluctuations of individual ion channels. The increase of the leak conductance starts after a delay period with burstlike fluctuations and finally leads to conductance values at least 3 orders of magnitude larger than before illumination. The increase after a minimum time of illumination continues in the dark (Fig. 6). In the case of lipid membranes, the phenomena have been interpreted via an accumulation of products of lipid peroxidation. According to Mirsky et al. (1991), the conductance starts to increase, if the concentration of polar decomposition products of the lipids exceeds a critical value. Charge transfer is believed to occur through porelike structures formed by the photoproducts.

Application of these ideas to the plasma membrane of OK-cells requires additional assumptions in order to understand the continuation of the conductance increase in the dark (after light-induced initiation, *cf.* Fig. 6). Two alternatives have been envisaged. The process of photodynamically induced lipid peroxidation might be maintained beyond the period of illumination (e.g., by way of action of long-living radical species). Alternatively, the formation (from the photoproducts as molecular constituents) of the new ionic pathways through the membrane might be a slow and rate-limiting process, which extends into the dark period following illumination. The structures underlying these pathways must

Table 2. Membrane potentials and relative permeabilities for different cations and anions

Pipette	Bath	$\Delta V'_Z$/mV	V_d/mV	V_m/mV	P_X/P_{Cl}	X	n
	Na 1 (19 Na^+, 14 Cl^-)	−19.7 ± 1.7	11.8	−7.9	1.5 ± 0.1	Na^+	4
	Na 2 (88 Na^+, 84 Cl^-)	−8.0 ± 0.6	3.1	−4.9	2.0 ± 0.2	Na^+	3
High-NaCl	TEA (139 TEA^+, 13 Na^+, 148 Cl^-)	−13.4 ± 0.4	−3.2	−16.6	0.30 ± 0.02	TEA^+	2
(158 Na^+ 154 (Cl^-)	Glutamate (151 Na^+, 4 Cl^-, 150 Glu^-)	−0.5 ± 1.3	8.7	8.2	0.17 ± 0.10	glu^-	5
	Choline (140 $choline^+$, 13 Na^+, 140 Cl^-)	−13.4 ± 0.7	−3.5	−16.9	0.29 ± 0.04	$choline^+$	5
	$CaCl_2$ (75 Ca^{2+}, 151 Cl^-, 1 Na^+)	0.6 ± 0.5	−4.2	−3.6			6

All potentials refer to the pipette electrode. For the composition of the solutions *see also* Table 1. The numbers represent concentrations in mM. $V_m = \Delta V_z$ is the zero-current potential difference corrected for the diffusion potential, V_d, at the tip of the pipette after disruption of the patch (*see* Materials and Methods). $\Delta V'_z$ corresponds to the value of ΔV_z before correction.

Table 3. Ion concentrations (mM) and relative permeabilities, P'_{Cl} and P'_{Ca}, of the photomodified plasma membrane (u = reduced membrane potential difference, *see* text)

$[Na^+]_{bath}$	$[Ca^{2+}]_{bath}$	$[Cl^-]_{bath}$
1	75	151
$[Na^+]_{pip}$	$[Ca^{2+}]_{pip}$	$[Cl^-]_{pip}$
158	1	154
P'_{Cl}	u	P'_{Ca}
0.59	−0.14	0.8

show comparatively small selectivity between monovalent and divalent cations and must also allow the permeation of larger monovalent cations and anions. The selectivity data of the present study (Table 2) show fundamental agreement with a previous analysis by Deuticke et al. (1989), who reported an appreciable permeability of the photodynamically induced leaks in human erythrocytes for polar solutes such as sucrose. Contrary to the present study, however, the authors preferred to interpret the enhanced leak on the basis of oxidative damage of membrane proteins. Lebedev et al. (1982) on the other hand reported an increased Ca^{2+} permeability of oxidized lipid membranes. Their results rather suggest that the enhanced Ca^{2+} permeability of the plasma membrane of OK-cells (*cf.* Table 3) is due to a modified lipid component.

In summary, both phenomena reported, the functional modification of ion channels and the increase of the leak conductance, may be explained as a result of photodynamically induced lipid peroxidation, though other alternatives cannot be excluded at present.

The photomodification of the maxi-K_{Ca} may contribute to certain physiological responses of the cell after a photodynamic challenge. The transient activation of the channels might trigger Ca^{2+}-dependent rescue mechanisms (Penning et al., 1992; Penning et al., 1993) or might stimulate the light avoidance reaction of paramecia observed in the presence of sensitizers (Saitow & Nakaoka, 1996). The maxi-K_{Ca} has been shown to control the arterial vessel tone (Brayden & Nelson, 1992). Therefore, its inactivation might contribute to the vasoconstrictive effects of the photodynamic therapy (Zhou, 1989; Ben-Hur & Orenstein, 1991; Chaplin, 1991).

Important physiological consequences of the photomodifications apply to the membrane potential, V_m, and to the Ca^{2+} permeability of the plasma membrane. The depolarization of V_m has been observed with different cellular systems and sensitizers (*see* Kunz & Stark, 1998). It is an early 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 (Specht & Rodgers, 1990; Kochevar et al., 1994). For the system investigated, the depolarization is caused by two different phenomena (*see* Kunz & Stark, 1998). The initial part of the depolarization was found to be due to the inactivation of the K^+ conductance of the membrane, while the final part was due to a nonspecific increase of the leak conductance. In the present part II of the study, both processes are analyzed at the microscopic level. Under normal physiological conditions, the membrane conductance, is largely determined by the small conductance (sK) K^+ channel (Kolb, 1990), which is shown to be inactivated by a photosensitized process (*cf.* Fig. 3). The leak conductance is found to have similar permeabilities for monovalent cations and anions of similar size (*see* Fig. 4 of part I and Table 2 of part II). It will therefore also contribute to the depolarization of V_m, as is easily shown by application of the Goldman-Hodgkin-Katz Equation (A1). Thus, the results of part II explain

and support the experimental results of the whole-cell study.

The second important physiological consequence may be attributed to the enhanced Ca^{2+} permeability of the photodynamically induced leak conductance. It may give rise to a flow of Ca^{2+} ions from the extracellular medium into the cell. The resulting increase of the cytoplasmic Ca^{2+} concentration has been repeatedly analyzed (Yonuschot, Vaughn & Novotny, 1992; Dellinger et al., 1994; Joshi et al., 1994; Gederaas et al., 1996; Hubmer et al., 1996) and has been suggested to be a trigger for further intracellular processes finally leading to photosensitized cell death (Ben-Hur & Dubbelman, 1993). So far, the increase of cytoplasmic Ca^{2+} has been explained via the activation of Ca^{2+} channels of the plasma membrane or via Ca^{2+} release from intracellular stores. Permeation through Ca^{2+} permeable, porelike structures formed by photoproducts of lipid peroxidation permits an alternative, simple, and straightforward explanation of this phenomenon.

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References

Adam, G., Läuger, P., Stark, G. 1995. Physikalische Chemie und Biophysik. Springer-Verlag, Berlin

Baron, A., Frieden, M., Bény, J.-L. 1997. Expoxyeicosatrienoic acids activate a high-conductance, Ca^{2+}-dependent K^+ channel on pig coronary artery endothelial cells. *J. Physiol.* **504:**537–543

Ben-Hur, E., Dubbelman, T.M.A.R. 1993. Cytoplasmic free calcium changes as a trigger mechanism in the response of cells to photosensitization. *Photochem. Photobiol.* **58:**890–894

Ben-Hur, E., Orenstein, A. 1991. The endothelium and red blood cells as potential targets in PDT-induced vascular stasis. *Int. J. Radiat. Biol.* **60:**293–301

Brayden, J.E., Nelson, M.T. 1992. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* **256:**533–535

Buettner, G.R., Kelley, E.E., Burns, C.P. 1993. Membrane lipid free radicals produced from L1210 murine leukemia cells by photofrin photosensitization: an electron paramagnetic resonance spin trapping study. *Cancer Res* **53:**3670–3673

Chaplin, D.J. 1991. The effect of therapy on tumour vascular function. *Int. J. Radiat. Biol.* **60:**311–325

Dellinger, M., Ricchelli, F., Moreno, G., Salet, C. 1994. Hematoporphyrin derivative (photofrin®) photodynamic action on Ca^{2+} transport in monkey kidney cells (CV-1). *Photochem. Photobiol.* **60:**368–372

Deuticke, B., Henseleit, U., Haest, C.W.M., Heller, K.B., Dubbleman, T.M.A.R. 1989. Enhancement of transbilayer mobility of a membrane lipid probe accompanies formation of membrane leaks during photodynamic treatment of erythrocytes. *Biochim. Biophys. Acta* **982:**53–61

Ebihara, S., Shirato, K., Harata, N., Akaike, N. 1995. Gramicidin-perforated patch recording: GABA response in mammalian neurons with intact intracellular chloride. *J. Physiol.* **484:**77–86

Favero, T.G., Zable, A.C., Abramson, J.J. 1995. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **270:**25557–25563

Gederaas, O.A., Thorstensen, K., Romslo, I. 1996. The effect of brief illumination on intracellular free calcium concentration in cells with 5-aminolevulinic acid-induced protoporphyrin IX synthesis. *Scand. J. Clin. Lab. Invest.* **56:**583–589

Girotti, A.W. 1990. Photodynamic lipid peroxidation in biological systems. *Photochem. Photobiol.* **51:**497–509

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hollunder-Reese, F., Mohrmann, M., Bleich, M., Greger, R. 1991. K^+-, Cl^-, and nonselective channels in opposum kidney cells. *Cell. Physiol. Biochem.* **1:**238–250

Holmberg, S.R., Cumming, D.V., Kusama, Y., Hearse, D.J., Poole-Wilson, P.A., Shattock, M.J., Williams, A.J. 1991. Reactive oxygen species modify the structure and function of the cardiac sarcoplasmic reticulum calcium-release channel. *Cardioscience* **2:**19–25

Horn, R., Marty, A. 1988. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* **92:**145–159

Hubmer, A., Hermann, A., Überriegler, K., Krammer, B. 1996. Role of calcium in photodynamically induced cell damage of human fibroblasts. *Photochem. Photobiol.* **64:**211–215

Joshi, P.G., Joshi, K., Mishra, S., Joshi, N.B. 1994. Ca^{2+} influx induced by photodynamic action in human cerebral glioma (U-87 MG) cells: possible involvement of a calcium channel. *Photochem. Photobiol.* **60:**244–248

Kelley, E.E., Buettner, G.R., Burns, C.P. 1997. Production of lipid-derived free radicals in L1210 murine leukemia cells is an early oxidative event in the photodynamic action of photofrin®. *Photochem. Photobiol.* **65:**576–580

Kochevar, I.E., Bouvier, J., Lynch, M., Lin, C.-W. 1994. Influence of dye and protein location on photosensitization of the plasma membrane. *Biochim. Biophys. Acta* **1196:**172–180

Kolb, H.-A. 1990. Ion channels in opossum kidney cells. *Renal Physiol. Biochem.* **13:**26–36

Koliwad, S.K., Kunze, D.L., Elliott, S.J. 1996. Oxidant stress activates a non-selective cation channel responsible for membrane depolarization in calf vascular endothelial cells. *J. Physiol.* **491:**1–12

Koufen, P., Zeidler, U., Stark, G. 1997. Photodynamic inactivation of ion channels formed by the polyene antibiotic amphotericin B in lipid membranes. *J. Photochem. Photobiol. B: Biol.* **38:**129–135

Kunz, L., Zeidler, U., Hägele, K., Przybylski, M., Stark, G. 1995. Photodynamic and radiolytic inactivation of ion channels formed by gramicidin A: oxidation and fragmentation. *Biochemistry* **34:**11895–11903

Kunz, L., Stark, G. 1997. Photodynamic membrane damage at the level of single ion channels. *Biochim. Biophys. Acta* **1327:**1–4

Kunz, L., Stark, G. 1998. Photofrin sensitized modifications of ion transport across the plasma membrane of an epithelial cell line. I. Electrical measurements at the whole-cell level. *J. Membrane. Biol.* 166:**part I**

Kunz, L. 1998. Photodynamische Wirkungen von Photofrin II auf Ionenkanäle und Leckleitfähigkeit der Plasmamembran. Doctorial thesis, University of Konstanz, Konstanz, Germany

Kyrozis, A., Reichling, D.B. 1995. Perforated patch recording with gramicidin A avoids artifactual changes in intracellular chloride concentration. *J. Neurosci. Methods* **57:**27–35

Lebedev, A.V., Levitsky, D.O., Loginov, V.A., Smirnov, V.N. 1982. The effect of primary products of lipid peroxidation on the trans-

membrane transport of calcium ions. *J. Mol. Cell. Cardiol.* **14:**99–103

Meves, H. 1994. Modulation of ion channels by arachidonic acid. *Prog. Neurobiol.* **43:**175–186

Mirsky, V.M., Stozhkova, I.N., Szitó. T.V. 1991. Photosensitized damage of bilayer lipid membrane in the presence of haematoporphyrin dimethylether, *J. Photochem. Photobiol. B: Biol.* **8:**315–324

Penning, L.C., Keirse, M.J.N.C., Van Steveninck, J., Dubbelman, T.M.A.R. 1993. Ca^{2+}-mediated prostaglandin E_2 induction reduces haematoporphyrin-derivative cytotoxicity of T24 human bladder transitional carcinoma cells in vitro. *Biochem. J.* **292:**237–240

Penning, L.C., Rasch, M.H., Ben-Hur, E., Dubbelman, T.M.A.R., Havelaar, A.C., Van der Zee, J., Van Steveninck, J. 1992. A role for the transient increase of cytoplasmic free calcium in cell rescue after photodynamic treatment. *Biochim. Biophys. Acta* **1107:**255–260

Petrou, S., Ordway, R.W., Kirber, M.T., Dopico, A.M., Hamilton, J.A., Walsh, J.V., Jr., Singer, J.J. 1995. Direct effects of fatty acids and other charged lipids on ion channel activity in smooth muscle cells. *Prostaglandins Leukotrienes and Essential Fatty Acids* **52:**173–178

Pooler, J.P. 1987. Photodynamic modification of excitable cell function. *In:* Light-activated pesticides. J.R. Heitz, editor. pp. 109–121. ACS symposium series 339, American Chemical Society, Washington, DC

Rae, J., Cooper, K., Gates, P., Watsky, M. 1991. Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods* **37:**15–26

Rokitskaya, T.I., Antonenko, Y.N., Kotova, E.A. 1993. The interaction of phthalocyanine with planar lipid bilayers. Photodynamic inactivation of gramicidin channels. *FEBS Lett.* **329:**332–335

Rokitskaya, T.I., Antonenko, Y.N., Kotova, E.A. 1996. Photodynamic inactivation of gramicidin channels: a flash-photolysis study. *Biochim. Biophys. Acta* **1275:**221–226

Saitow, F., Nakaoka, Y. 1996. Photodynamic action of methylen blue on the paramecium membrane. *Photochem. Photobiol.* **63:**868–873

Specht, K.G., Rodgers, M.A.J. 1990. Depolarization of mouse myeloma cell membranes during photodynamic action. *Photochem. Photobiol.* **51:**319–324

Sträßle, M., Stark, G. 1992. Photodynamic inactivation of an ion channel: Gramicidin A. *Photochem. Photobiol.* **55:**461–463

Tarr, M., Valenzeno, D.P. 1991. Modification of cardiac ionic currents by photosensitizer-generated reactive oxygen. *J. Mol. Cell. Cardiol.* **23:**639–649

Twitchell, W.A., Peña, T.L., Rane, S.G. 1997. Ca^{2+}-dependent K^+ channels in bovine adrenal chromaffin cells are modulated by lipoxygenase metabolites of arachidonic acid. *J. Membrane Biol.* **158:**69–75

Ubl, J. 1992. Ionentransportsysteme in einer Nierenzellinie (OK-Zellen) und deren Beteiligung an der Volumenregulation. Doctoral thesis, University of Konstanz, Konstanz, Germany

Ubl, J., Murer, H., Kolb, H.-A. 1988. Ion channels activated by osmotic and mechanical stress in membranes of opossum kidney cells. *J. Membrane Biol.* **104:**223–232

Valenzeno, D.P. 1987. Photomodification of biological membranes with emphasis on singlet oxygen mechanisms. *Photochem. Photobiol.* **46:**147–160

Valenzeno, D.P., Tarr, M. 1995. Mechanisms of cellular photomodification. *In:* Light-activated pest control. J.R. Heitz, editor. pp. 24–33. ACS symposium series 616, American Chemical Society, Washington, DC

Xiong, H., Buck, E., Stuart, J., Pessah, I.N., Salama, G., Abramson, J.J. 1992. Rose bengal activates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **292:**522–528

Yonuschot, G., Vaughn, J.M., Novotny, J.F. 1992. Intracellular calcium during photodynamic permeabilization of cardiomyocytes. *J. Mol. Cell. Cardiol.* **24:**1079–1088

Zhou, C. 1989. Mechanisms of tumor necrosis induced by photodynamic therapy. *J. Photochem. Photobiol. B: Biol.* **3:**299–318

Appendix

Estimation of the Ca^{2+} Permeability of Photomodified Inside-out Patches

In the presence of monovalent cations and anions the membrane potential may be calculated using the classical Goldman-Hodgkin-Katz Equation

$$V_m = \Psi_{pip} - \Psi_{bath} = (RT/F) \cdot ln \frac{\sum \frac{P_{cation}}{P_{Cl}} \cdot c^{bath}_{cation} + \sum \frac{P_{anion}}{P_{Cl}} \cdot c^{pip}_{anion}}{\sum \frac{P_{cation}}{P_{Cl}} \cdot c^{pip}_{cation} + \sum \frac{P_{anion}}{P_{Cl}} \cdot c^{bath}_{anion}}. \quad \text{(A1)}$$

V_m is obtained from the Nernst-Planck Equation

$$\Phi_i = -D_i \cdot (d[\mathrm{i}]/dx + z_\mathrm{i} \cdot F \cdot [i]/(R \cdot T) \cdot d\psi/dx), \quad \text{(A2)}$$

with Φ_i = flux of ion i, $[i]$ = concentration of the ion, z_i = valency, D_i = diffusion coefficient, x = space coordinate, F = Faraday constant, ψ = potential.

Integration of (A2) under appropriate assumptions yields the current, I_i, carried by the species i (Adam et al., 1995)

$$I_i = z_i \cdot F \cdot \Phi_i = z_i^2 \cdot F \cdot P_i \cdot u \cdot ([i]_a - [i]_i \cdot \exp(z_i u))/(1 - \exp(z_i u)). \quad \text{(A3)}$$

Under zero-current conditions,

$$I = \sum_i I_i = 0. \quad \text{(A4)}$$

In the presence of monovalent cations and anions, after combination of Eqs. (A3) and (A4) and rearrangement, Eq. (A1), the Goldman-Hodgkin-Katz Equation is obtained. In the presence of divalent ions, the resulting equation cannot be solved for V_m. It allows, however, to calculate relative permeabilities. If the aqueous solutions only contain the ion species Na^+, Ca^{2+}, and Cl^-, Eq. (1) is obtained (*see* Results). In this case the ratio $P'_{Ca} = P_{Ca} / P_{Na}$ is a function of $P'_{Cl} = P_{Cl} / P_{Na}$ and of the experimentally determined zero-current potential difference, $V_m = \Delta V_z$ (*see* Materials and Methods).

The procedure provides, however, only rough estimates for the permeability ratios. This holds in view of the stringent assumptions used for the derivation of Eq. (1). In addition to the usual limitations of Eq. (A1), the existence of interfacial potentials at the membrane/water interfaces, as well as Ca^{2+}-binding to the membrane, has been neglected.