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Photomodification of the Electrical Properties of the Plasma Membrane: A Comparison Between 6 Different Membrane-Active Photosensitizers

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Abstract. The present study deals with photomodification of the electrical properties of the plasma membrane of an epithelial cell line (opossum kidney (OK) cells). The effect of photofrin II (previously investigated) is compared with that of 5 other membrane-active sensitizers: sulfonated Zn-phthalocyanine, merocyanine 540, rose bengal, methylene blue and protoporphyrin IX (an endogenous sensitizer induced by addition of its biosynthetic precursor 5-aminolaevulinic acid). The study was performed in order to investigate whether photomodification of the ion transport properties of the plasma membrane by membrane-active sensitizers is a general and early event in cellular photosensitization. The changes in the electrical properties were monitored by application of the whole-cell and the inside-out configuration of the patch-clamp technique.

Illumination in the presence of the compounds (apart from merocyanine 540) gave rise to similar changes of the electrical properties of the membrane: depolarization of the membrane potential, inactivation of a large-conductance, Ca^{2+}-dependent K^{+}-channel (maxi-K_{Ca}), and a strong increase of the leak conductance of the membrane. This similarity indicates the general character of the functional photomodifications by membrane-active sensitizers previously reported for photofrin II.

Key words: Photosensitization — Patch-clamp — OK-cells — Membrane potential — Ion channel — Maxi K — Leak conductance

Introduction

Illumination with visible light in the presence of certain photosensitizers is known to induce strong cellular damage finally leading to cell death by apoptosis or necrosis. These findings have led to a series of clinical applications such as the photodynamic therapy [3]. For many of the photosensitizers used at these applications, the plasma membrane and the membranes of cellular organelles (mitochondria) have been suggested to act as important sites of photodynamically induced cellular damage [12, 13]. Due to their lipophilic nature, these sensitizers partition into nonpolar media such as the interior of a biological membrane. As a consequence of this enrichment, early events in the photodynamically induced cellular damage have been observed which clearly indicate photomodification of cellular membranes and of their transport systems: the depolarization of the membrane potential and an increase in the cytoplasmic free calcium concentration [2, 16]. Both phenomena result from changes in ion transport across biological membranes, which may be investigated by electrophysiological methods such as the patch-clamp technique. Studies of this kind have been mainly performed for excitable cells with rose bengal as sensitizer [17–20] and for an epithelial cell line in the presence of photofrin II [5–7]. Photomodification of the respective plasma membranes was observed to inactivate specific potassium, sodium, and calcium currents and to increase the basic (nonspecific) leak conductance of the membrane [17–20]. The increase of the latter was found to cover up to three orders of magnitude and to include an enhanced permeation of Ca^{2+}-ions [6, 7]. In addition, inactivation of ionic currents was shown at the level of single ion channels [5]. These findings allow to explain the two early, membrane associated, photodynamic events mentioned above, i.e., the depolarization of the membrane potential and the increase of the cytoplasmic Ca^{2+} concentration [7].

Despite the relevance of membrane photomodifications, the corresponding studies were so far limited to a few selected systems. Therefore, the question arises whether photodynamically induced changes of ion trans-

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port processes are a general event in cellular photosensitization. The present communication is intended to contribute to this issue by comparing the photodynamic effects of several well-known, chemically different, membrane-active photosensitizers at the same system: the plasma membrane of an epithelial cell line obtained from the opossum kidney (OK). This cell line has previously been used to study photomodification by photofrin II [5–7]. The effect of this sensitizer is compared with those of sulfonated Zn-phthalocyanine, merocyanine 540, rose bengal, methylene blue and protoporphyrin IX (an endogenous sensitizer induced by addition of its biosynthetic precursor 5-aminolaevulinic acid).

Materials and Methods

Electrophysiology

The effect of visible light on the electrical characteristics of the plasma membrane of OK-cells was investigated by application of the patch-clamp method in the presence of the photosensitizers mentioned below. The study was performed by making use of the whole-cell and the inside-out configurations of this technique as described in detail in previous publications [5, 6]. OK-cells, kindly supplied by Dr. H. Murer (Zürich, Switzerland), were maintained in culture [5]. The decay of their zero-current potential, V_m, was determined in the current-clamp mode of an EPC-9 patch-clamp amplifier by use of the software Pulse (HEKA Elektronik, Lambrecht, Germany). The whole-cell recordings were obtained using a high-NaCl solution (in mM, 140 NaCl, 4 KCl, 1 $CaCl_2$) in the bath and a high-KCl solution (in mM, 135 KCl, 20 NaCl, 1 $CaCl_2$) in the pipette. The pipette solution additionally contained 2 mM EGTA to ensure a free Ca^{2+}-concentration of about 0.1 μM. All solutions additionally contained (in mM) 1 $MgCl_2$, 18 (or 10) glucose and 20 (or 10) HEPES. The pH of the solutions was adjusted to 7.4.

The inside-out configuration of the patch-clamp technique was applied in order to study single channel fluctuations of the large-conductance, Ca^{2+}-dependent K^+-channel (maxi-K_{Ca}). Measurements were performed by using the high-KCl solution for both, bath and pipette (in the absence of EGTA, however, to activate the Ca^{2+}-dependent channel). The same technique was applied to study photomodification of the leak conductance. In this case, the high-NaCl solution was used for bath and pipette (to reduce a possible contribution of K^+-channels). Changes of the leak conductance may also be detected by the whole-cell technique [5]. The use of the inside-out configuration is, however, preferred since the measurements can be performed in the absence of active ion channels (without the use of blocking substances).

Application of the Photosensitizers

The type and concentration of the photosensitizers were as follows: 2 μg/ml photofrin II (Quadra Logic Technologies, Kattendijke, Netherlands), 1 μM of a water soluble mixture of single- and double-sulfonated Zn-phthalocyanine ($ZnPcS_{1/2}$) prepared according to the procedure of Ali et al. [1], 5 μM merocyanine 540 (MC540; Sigma, St. Louis, MO), 1 μM rose bengal (Aldrich, Milwaukee, WI), 10 μM methylene blue (MB; Fluka, Buchs, Switzerland), and 1 mM 5-aminolaevulinic acid (ALA; Merck, Darmstadt, Germany), the biosynthetic precursor of protoporphyrin IX. In the case of photofrin II, $ZnPcS_{1/2}$, MC540, and (partly) methylene blue, OK-cells were incubated for 1 hr in the high-NaCl solution containing the sensitizer concentrations indicated. Thereafter, cells were stored in the same solution without sensitizer. Electrophysiological experiments were performed up to three hours after incubation. For ALA, the incubation time was increased up to 18 hr (applied in Figs. 1 and 2). Contrary to the sensitizers mentioned before, incubation was performed in the culture medium in an incubator at 37°C and 5% CO_2 (*see* [6] for details). This was done in order to allow cellular synthesis of protoporphyrin IX under the normal conditions of cell culture.

In the case of rose bengal, the sensitizer was added to the bath solution (in the form of an aqueous stock solution of 1 mM concentration) a few minutes before the measurement was performed (final concentration 1 μM). The same procedure was partly also applied for some of the other sensitizers.

Illumination

After establishment of the patch-clamp configuration and a period of control measurements, cells were illuminated with a laser and the photomodification of electrical properties was observed during illumination. The light was focused via fiber optics onto the cells under an angle of 45°. Three different laser types were applied: HeNe (632.8 nm, Melles Griot, Carlsbad, CA) for photofrin II and ALA-induced protoporphyrin IX, HeNe (543.5 nm, Melles Griot) for MC540 and rose bengal, and a diode laser (670 nm, Coherent, Auburn, CA) for $ZnPcS_{1/2}$ and methylene blue. The mean irradiance was 5000 W/m^2, 1500 W/m^2, and 550 W/m^2, respectively.

General

All experiments were performed at room temperature and were repeated at least 3 times. Throughout the incubation and the entire experiment the cells were kept in the dark to avoid unwanted photodynamic reactions. The software Fig.P (Fig.P Software Corp., Durham, NC) was used for data reduction and analysis.

Abbreviations

OK	opossum kidney
$ZnPcS_{1/2}$	mono-/disulfonated Zn-phthalocyanine
MC540	merocyanine 540
MB	methylene blue
ALA	5-aminolaevulinic acid

Results and Discussion

The electrical conductance of the plasma membrane (apart from minor contributions of electrogenic ion pumps) is largely determined by its specific ion channels and by its basic (leak) conductance. Comparison of the various photosensitizers was performed on the basis of three different kinds of measurements: the decay of the zero current potential, V_m, (Fig. 1), the increase of the leak conductance, G_m, (Fig. 2), and the inactivation of the large-conductance, Ca^{2+}-dependent K^+-channel (maxi-K_{Ca}) (Fig. 3). The maxi-K_{Ca} was chosen as a model for specific ion channels. Its photomodification by two of the sensitizers (i.e., rose bengal (Fig. 3*a*)

and $ZnPcS_{1/2}$ (Fig. 3*b*)) was investigated and compared with the effect of photofrin II [7]. In all three cases, the typical current fluctuations of this ion channel—characterized by brief transitions from the open to the closed channel state—disappear after the start of illumination, i.e., the current level was found to agree with that in the absence of the channel. In some cases (*see* last trace of Fig. 3*b*), the onset of the increase of the leak conductance becomes visible, which was studied in more detail in the absence of active ion channels (Fig. 2).

The decay of the membrane potential and the strong increase of the leak conductance during illumination were found for all photosensitizers investigated apart from MC540 (Fig. 2). Differences in the effectiveness were observed, however. These are partly due to the different light intensities applied, due to the different absorption coefficients of the sensitizers, and due to the different sensitizer concentrations in the membrane. In order to compensate for these differences, i.e., to observe modifications on the same time scale, measurements were performed after different incubation times, at different concentrations or by addition of the sensitizer directly to the bath solution (*see* Materials and Methods).

The following control experiments were performed: a) Illumination of the membrane with either of the three lasers (with wavelengths 543.5 nm, 632.8 nm, and 670 nm) in the absence of any photosensitizer. b) Modification of the electrical properties by addition of the sensitizers in the absence of light. At all control experiments performed, the electrical membrane properties were found to remain unaffected, i.e., their modification requires both, the presence of one of the sensitizers and of light.

In the case of ALA, the biosynthetic precursor of the sensitizer protoporphyrin IX, an increase of the leak conductance was only observed after a comparatively long incubation time of 18 hr (Fig. 2*f*), while there was a significant decrease of the membrane potential already after an incubation time of 0.5 hr (data not shown). Obviously, V_m is a sensitive sensor of photodynamically induced membrane damage. The decay of V_m is due to a combined effect of the inactivation of K^+-selective ion channels (different from the maxi-K_{Ca}) and of the increase of the nonselective leak conductance, as was shown in detail for photofrin II [6]. We suspect that it is the inactivation of these K^+-channels, which primarily causes the decay of V_m observed after short incubation times with ALA. This assumption is supported by the finding that photodynamically induced inactivation of K^+-channels (by photofrin II) precedes the increase in the leak conductance [7].

Rose bengal showed no light-induced effects if measurements were performed after the incubation procedure. This sensitizer, however, was found to be very efficient if present (at 1 μM concentration) in the bath

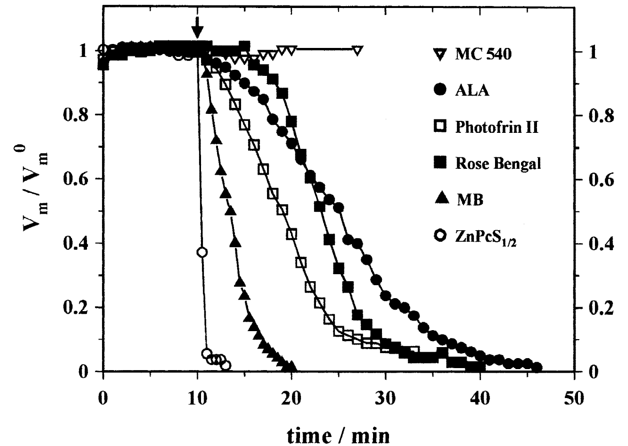

Fig. 1. The decay of the membrane potential during illumination of OK-cells in the presence of different photosensitizers. Illumination was started at the arrow. $V_m{}^0$ was in the range of −65 mV up to −85 mV in each case. The data were obtained using the whole-cell configuration of the patch-clamp technique. For concentrations of the sensitizers and the incubation procedure *see* Materials and Methods. The data represent typical observations which were repeated at least 3 times.

solution during the measurements. An enhancement of the efficiency by this method was also observed in the case of methylene blue. The light-induced increase of the leak conductance was 1–2 orders of magnitude if this sensitizer was added via the incubation procedure, but up to 3 orders of magnitude if methylene blue was present in the bath solution during the measurement (as shown in Fig. 2*e*). The increase in the efficiency is presumably caused by a more pronounced sensitizer enrichment in the plasma membrane.

Virtually no effect of visible light was observed after incubation with merocyanine 540 (c.f. Figs. 1 and 2) or after addition of this substance to the bath solution (data not shown), through this sensitizer—which has been used for specific photodynamic elimination of leukemia cells and enveloped viruses in bone marrow grafts and blood transfusion products—has clearly been characterized as membrane-active [9–11, 14, 15]. The binding affinity of MC540 to the plasma membrane has, however, been found to depend strongly on the cell type. High affinity binding has been observed for electrically excitable cells, for tumor cells and for enveloped viruses, while non-excitable cells show low affinity (see [15] for a review). No satisfactory interpretation for the specificity of membrane binding appears to be available at present. There is, however, another factor which might contribute to the comparatively low efficiency of MC540, namely its low quantum yield of singlet oxygen formation (≤0.04 in lipid vesicles [4]). In spite of this low yield, $^1O_2$ has been suggested as an important intermediate in MC540-induced photodynamic membrane effects [10]. If this is correct, light-induced membrane modifications would be visible in the presence of com-

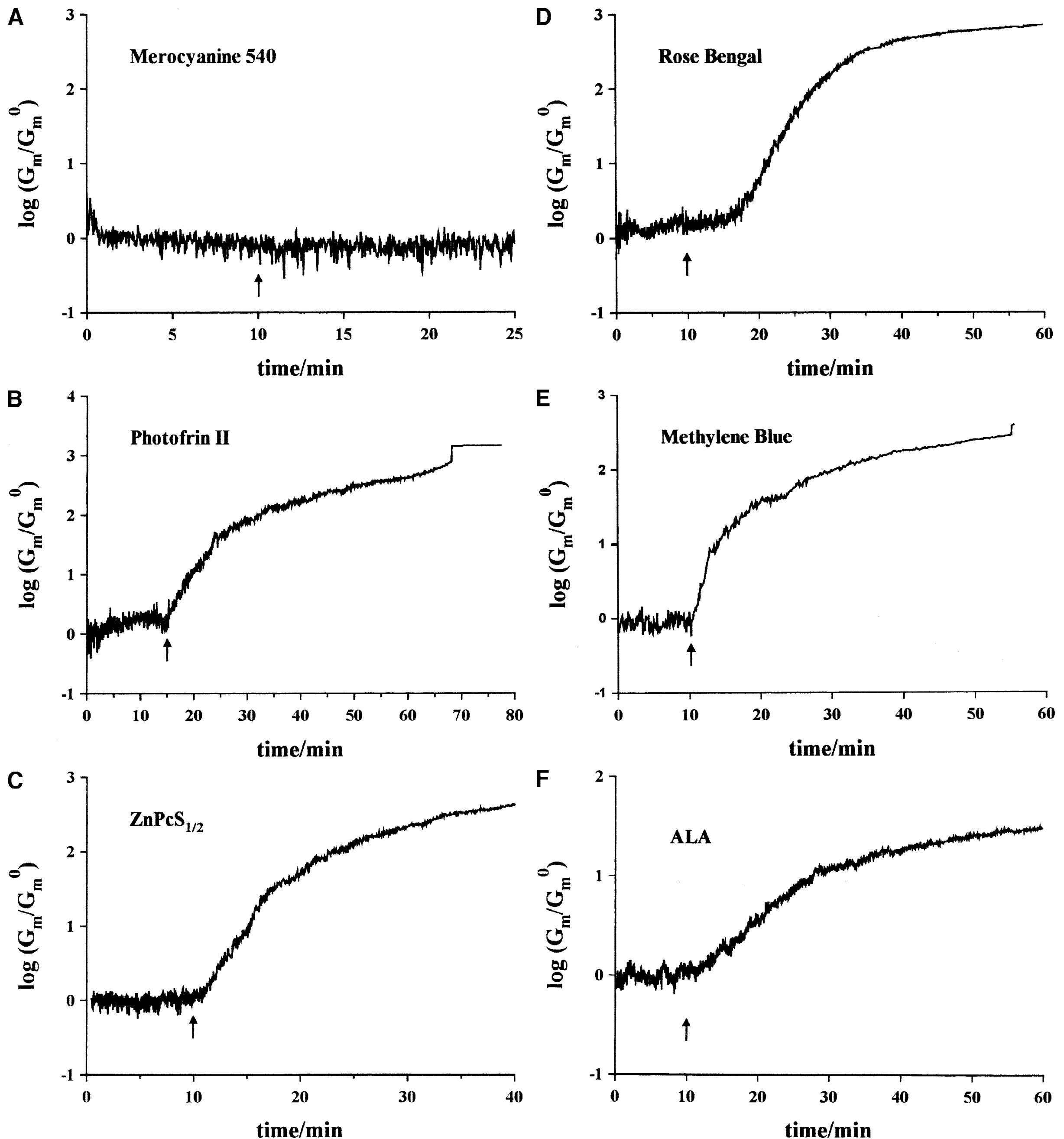

Fig. 2. The light-induced change of the leak conductance investigated in the presence of the different photosensitizers. The data were obtained by using the inside-out configuration of the patch-clamp technique in the absence of active ion channels. The arrows indicate the onset of illumination. G_m^0, the leak conductance before illumination, was between 50 and 150 pS in all cases. For concentrations of the sensitizers and the incubation procedure *see* Materials and Methods. The data represent typical observations which were repeated at least 3 times.

paratively high membrane concentrations only. The partition coefficient of MC540 between membrane and water was found to depend on structural membrane properties as well as on the membrane potential, negative potentials being unfavorable [9, 10]. If applied to the plasma membrane of OK-cells, these findings (in view of the negligible light effects observed) would predict that the partition coefficient of MC540 is inadequate to compensate its low quantum yield of singlet oxygen formation.

Ideally, comparison of different sensitizers should be performed on the basis of the concentration of the generated reactive species (singlet oxygen etc.). This, however, requires knowledge of the membrane concen-

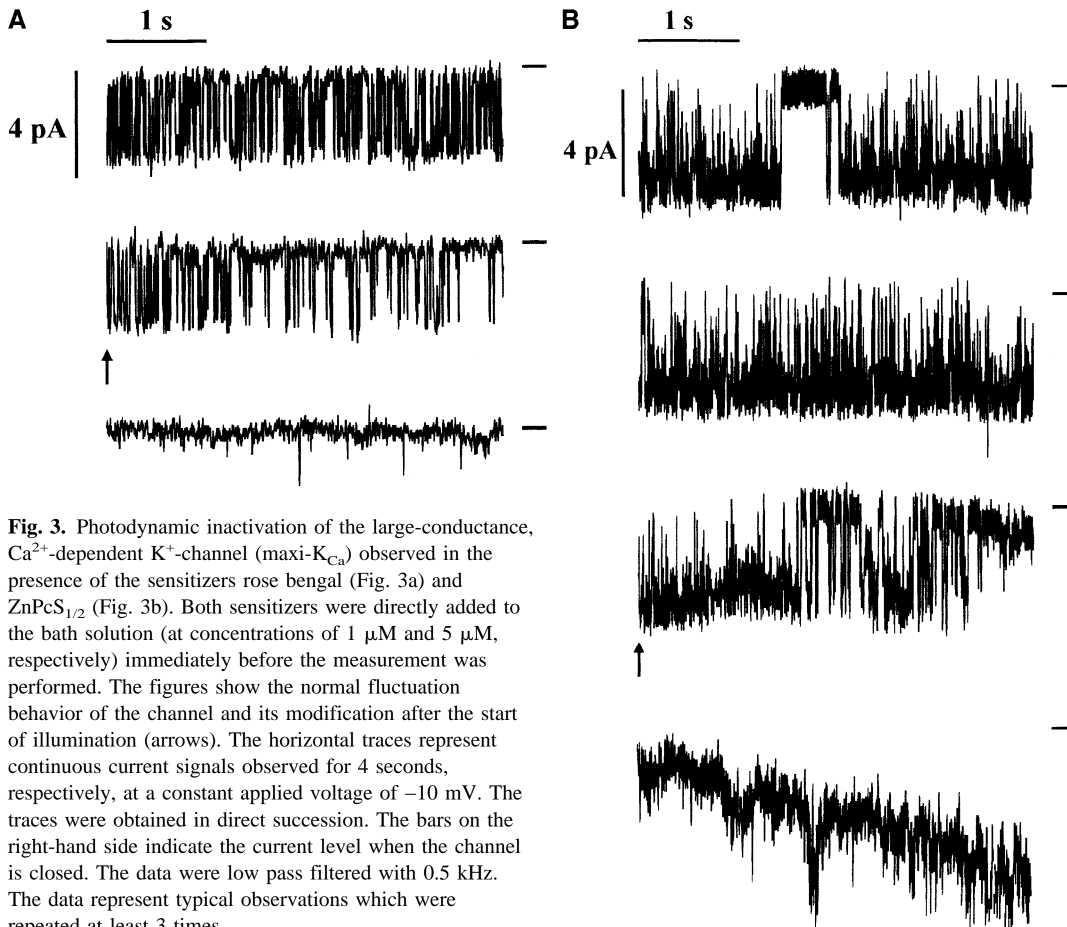

Fig. 3. Photodynamic inactivation of the large-conductance, Ca^{2+}-dependent K^{+}-channel (maxi-K_{Ca}) observed in the presence of the sensitizers rose bengal (Fig. 3a) and $ZnPcS_{1/2}$ (Fig. 3b). Both sensitizers were directly added to the bath solution (at concentrations of 1 μM and 5 μM, respectively) immediately before the measurement was performed. The figures show the normal fluctuation behavior of the channel and its modification after the start of illumination (arrows). The horizontal traces represent continuous current signals observed for 4 seconds, respectively, at a constant applied voltage of −10 mV. The traces were obtained in direct succession. The bars on the right-hand side indicate the current level when the channel is closed. The data were low pass filtered with 0.5 kHz. The data represent typical observations which were repeated at least 3 times.

tration of singlet oxygen. The relation between the sensitizer concentrations and those of reactive species has been determined in homogeneous solutions such as water and (in part) in artificial lipid membranes (see ref [4] and the literature cited therein). Reliable values for the plasma membrane of the cell (at least to our knowledge) have not been available so far. Comparison of the membrane damage on the basis of the singlet oxygen concentrations in water would be misleading. This follows from our recent finding that reactive species generated directly in the membrane are far more efficient in the generation of membrane damage than reactive species generated in water [8]. It is the special property of membrane active sensitizers that (contrary to water soluble sensitizers) they are able to deliver singlet oxygen (as well as other reactive species) close to the locus where the damage will be observed. Unfortunately, however, the relation between the membrane concentrations of the sensitizers and of the reactive species—as a function of the sensitizer concentration in water—has not been established so far. In view of these difficulties we decided to restrict our study to a qualitative analysis. The aim of our investigation was to compare the action of the sensitizers on the basis of their modification of the electrical properties of the plasma membrane. The functional consequences accompanying illumination of the membrane were found to be largely identical for 5 of the 6 sensitizers: the depolarization of the membrane potential, a strong enhancement of the basic leak conductance, and the inactivation of an ion selective channel (maxi-K_{Ca}). These phenomena were already described in our previous, more detailed investigation of photofrin II-induced functional changes of the plasma membrane of OK-cells [7]. The results of the present communication suggest that the photodynamic membrane modifications described in previous studies may be generalized for most membrane-active photosensitizers.

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References

1. Ali, H., Langlois, R., Wagner, J.R., Brasseur, N., Paquette, B., van Lier, J.E. 1988. Biological activities of phthalocyanines—X. Syntheses and analyses of sulfonated phthalocyanines. *Photochem. Photobiol.* **47:**713–717
2. Ben-Hur, E., Dubbelman, T.M.A.R. 1993. Cytoplasmic free cal-

cium changes as a trigger mechanism in the response of cells to photosensitization. *Photochem. Photobiol.* **58:**890–894
3. Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbelik, M., Moan, J., Peng, Q. 1998. Photodynamic therapy. *J. Nat. Cancer Inst.* **90:**889–905
4. Krieg, M. 1992. Singlet oxygen production and fluorescence yields of merocyanine 540: a comparative study in solution and model membrane systems. *Biochim. Biophys. Acta* **1105:**333–335
5. Kunz, L., Stark, G. 1997. Photodynamic membrane damage at the level of single ion channels. *Biochim. Biophys. Acta* **1327:**1–4
6. Kunz, L., Stark, G. 1998. Photofrin II 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:**179–185
7. Kunz, L., Stark, G. 1998. Photofrin II sensitized modifications of ion transport across the plasma membrane of an epithelial cell line. II. Analysis at the level of membrane patches. *J. Membrane Biol.* **166:**187–196
8. Kunz, L., v. Weizsäcker, P., Mendez, F., Stark, G. 1999. Radiolytic and photodynamic modifications of ion transport through the plasma membrane of OK cells: a comparison. *Int. J. Radiat. Biol.* **75:**1029–1034
9. Lagerberg, J.W.M., Kallen, K.-J., Haest, C.W.M., VanSteveninck, J., Dubbelman, T.M.A.R. 1995. Factors affecting the amount and the mode of merocyanine 540 binding to the membrane of human erythrocytes. A comparison with the binding to leukemia cells. *Biochim. Biophys. Acta* **1235:**428–436
10. Lagerberg, J.W.M., VanSteveninck, J., Dubbelman, T.M.A.R. 1998. Effect of membrane potential on the binding of merocyanine 540 to human erythrocytes. *Photochem. Photobiol.* **67:**729–733
11. Lin, F., Girotti, A.W. 1993. Photodynamic action of merocyanine 540 on leukemia cells: iron-stimulated lipid peroxidation and cell killing. *Arch. Biochem. Biophys.* **300:**714–723
12. Moore, J.V., West, C.M.L., Whitehurst, C. 1997. The biology of photodynamic therapy. *Phys. Med. Biol.* **42:**913–935
13. Penning, L.C., Dubbelman, T.M.A.R. 1994. Fundamentals of photodynamic therapy: cellular and biochemical aspects. *Anti-Cancer Drugs* **5:**139–146
14. Seiber, F. 1987. Elimination of residual tumor cells from autologous bone marrow grafts by dye-mediated photolysis: Preclinical data. *Photochem. Photobiol.* **46:**71–76
15. Sieber, F. 1987. Merocyanine 540. *Photochem. Photobiol.* **46:** 1035–1042
16. Specht, K.G., Rodgers, M.A.J. 1991. Plasma membrane depolarization and calcium influx during cell injury by photodynamic action. *Biochim. Biophys. Acta* **1070:**60–68
17. Tarr, M., Valenzeno, D.P. 1991. Modification of cardiac ionic currents by photosensitizer-generated reactive oxygen. *J. Mol. Cell Cardiol.* **23:**639–649
18. Tarr, M., Valenzeno, D.P. 1998. Photomodification of cardiac membrane: Chaotic currents and high conductance states in isolated patches. *Photochem. Photobiol.* **68:**353–360
19. Valenzeno, D.P., Tarr, M. 1991. Membrane modification of cardiac myocytes: potassium and leakage currents. *Photochem. Photobiol.* **53:**195–201
20. Valenzeno, D.P., Tarr, M. 1998. GH_3 cells, ionic currents and cell killing: Photomodification by rose bengal. *Photochem. Photobiol.* **68:**519–526