Photosensitizer Binding to Lipid Bilayers as a Precondition for the Photoinactivation of Membrane Channels

Tatyana I. Rokitskaya,* Michael Block,† Yuri N. Antonenko,* Elena A. Kotova,* and Peter Pohl†
*A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia, and
†Martin-Luther-Universität, Medizinische Fakultät, Institut für Medizinische Physik und Biophysik, 06097 Halle, Germany

ABSTRACT The photodynamic activity of sulfonated aluminum phthalocyanines ($AlPcS_n$, $1 \leq n \leq 4$) was found to correlate with their affinity for membrane lipids. Adsorbing to the surface of large unilamellar vesicles (LUVs), aluminum phthalocyanine disulfonate induced the highest changes in their electrophoretic mobility. $AlPcS_2$ was also most efficient in mediating photoinactivation of gramicidin channels, as revealed by measurements of the electric current across planar lipid bilayers. The increase in the degree of sulfonation of phthalocyanine progressively reduced its affinity for the lipid bilayer as well as its potency of sensitizing gramicidin channel photoinactivation. The portion of photoinactivated gramicidin channels, α, increased with rising photosensitizer concentration up to some optimum. The concentration at which α was at half-maximum amounted to 80 nM, 30 nM, 200 nM, and 2 μM for $AlPcS_1$, $AlPcS_2$, $AlPcS_3$, and $AlPcS_4$, respectively. At high concentrations α was found to decrease, which was attributed to quenching of reactive oxygen species and self-quenching of the photosensitizer triplet state by its ground state. Fluoride anions were observed to inhibit both $AlPcS_n$ ($2 \leq n \leq 4$) binding to LUVs and sensitized photoinactivation of gramicidin channels. It is concluded that photosensitizer binding to membrane lipids is a prerequisite for the photodynamic inactivation of gramicidin channels.

INTRODUCTION

It is generally accepted that photomodification of biological membranes is one of the critical processes leading to cellular damage upon photodynamic treatment of living cells (Valenzeno and Tarr, 1991). Photomodification of channel-forming proteins (Valenzeno and Tarr, 1991; Tarr et al., 1995; Holmberg et al., 1991; Kunz and Stark, 1997, 1998a,b), initiated by irradiation with visible light in the presence of a photosensitizer, precedes the induction of nonspecific leakage conductance associated with photosensitized lipid peroxidation (Girotti, 1990; Ehrenberg et al., 1993).

Association of sensitizers with the membrane surface is expected to enhance the effectiveness of the photodynamic action on membrane components for several reasons. First, oxygen concentrations in lipid membranes are supposed to be approximately an order of magnitude greater than in the bulk aqueous solution. Second, the singlet oxygen lifetime and the photosensitizer triplet-state lifetime are reported to be longer in membranes relative to aqueous solution (Valenzeno, 1987; Howe and Zhang, 1998; Krasnovsky, 1998; Ehrenberg et al., 1998). The third reason, and probably the most important, is that the activity of singlet oxygen and other reactive oxygen species is confined by their mean diffusion distances. In fact, for a variety of sensitizers, the photosensitizing efficacy has been shown to correlate with their membrane association ability (Valenzeno and Tarr, 1991). This is particularly true for a class of phthalocyanine sensitizers, the hydrophobicity of which can be modulated by the degree of sulfonation (Paquette et al., 1988; Berg et al., 1989a). Although the increase in the degree of sulfonation is accompanied by a decrease in phototoxicity, the latter cannot be attributed to a diminishing of the partition coefficient alone because differences in cellular uptake are also involved (Paquette et al., 1991). For example, the uptake of monosulfonated aluminum phthalocyanine is 10-fold higher than the cellular uptake of tetrasulfonated aluminum phthalocyanine (Berg et al., 1989b). Furthermore, $AlPcS_4$ and $AlPcS_3$ are suggested to be in different intracellular locations than $AlPcS_2$ and $AlPcS_1$, a circumstance that also affects phototoxicity (Berg et al., 1989a). Consequently, to establish unambiguously a correlation between membrane binding and photodynamic efficiency, investigations of simple model membrane systems are required. The quantum yield of reactive oxygen species generation has been shown to depend negligibly on the degree of phthalocyanine sulfonation (Fernandez et al., 1997, and references therein), except for $AlPcS_1$, which is in line with the data on the triplet quantum yield and lifetime obtained for different sulfonated aluminum phthalocyanines (Foley et al., 1997). In certain cases, e.g., for erythrocyte photohemolysis, it is suggested that direct binding of phthalocyanine to some membrane proteins is a prerequisite for their photomodification (Ben-Hur et al., 1991, 1993).

However, with respect to the great variety of membrane proteins that are reported to undergo photodamage, it seems to be unlikely that specific binding is involved in all cases. Actually, the lifetime of reactive oxygen species generated

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Address reprint requests to Dr. Elena A. Kotova, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia. Tel.: +7095 9395360; Fax: +7095-9393181; E-mail: kotova@genebee.msu.su; or Dr. Peter Pohl, Martin-Luther-Universität, Medizinische Fakultät, Institut für Medizinische Physik und Biophysik, 06097 Halle, Germany. Tel.: +49-345-5571243; Fax: +49-345-5571632; E-mail: peter.pohl@medizin.uni-halle.de.

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by a photosensitizer (in particular, singlet oxygen) is ~3 μs in aqueous solution (Rodgers and Snowden, 1982; Krasnovsky, 1998) and is estimated to be longer than 7 μs in lipid membranes (Krasnovsky, 1998; Ehrenberg et al., 1998). Before reacting with a membrane-bound protein, encountering a quenching agent, or decaying from a variety of radiative and nonradiative processes, singlet oxygen can diffuse on the order of 100 nm (Valenzeno and Tarr, 1991). Consequently, the association with membrane lipids is proposed to be sufficient for a photosensitizer to exert a photodynamic effect on membrane proteins. The latter can be selectively studied if lipid peroxidation is minimized by using fully saturated lipids in model systems (Stozhkova et al., 1992; Rokitskaya et al., 1993). Here we address this issue, using the recently discovered phenomenon of gramicidin channel photoinactivation in planar bilayer lipid membranes (Straessle and Stark, 1992; Rokitskaya et al., 1993, 1996, 1997; Kunz et al., 1995). This model system offers remarkable advantages for studies of the photodynamic effect of different photosensitizers:

1. The activity of the latter can be measured at a very low protein-lipid ratio, because the pentadecapeptide gramicidin A is known to be a highly potent channel former (Hladky and Haydon, 1984; Koeppe and Andersen, 1996; Andersen et al., 1999).

2. The photosensitized suppression of the gramicidin-mediated current across bilayer lipid membrane (BLM) has proved to be specific, as it is caused by selective damage of tryptophan residues located at the channel entrance (Straessle and Stark, 1992).

In the present study, the efficacy of aluminum phthalocyanines in provoking the photodynamic inactivation of gramicidin channels in BLMs is found to correlate with their capability to adsorb to lipid bilayers. It is concluded that it is the membrane-bound phthalocyanine that mediates gramicidin photoinactivation.

MATERIALS AND METHODS

Planar bilayer membranes

BLMs were formed from a solution of 2% diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique (Mueller et al., 1963) on a 0.55-mm-diameter hole in a Teflon partition separating two aqueous compartments. The bathing aqueous solutions contained 1 M KCl, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 10 mM Tris, and 10 mM β-alanine (pH 7.0). At both sides of the BLM, gramicidin (Sigma, St. Louis, MO) was added from a stock solution in ethanol to the bathing solutions that were permanently stirred. The channel former incorporated spontaneously into the membrane. The membrane conductivity usually reached steady state within 15 min after the addition of gramicidin.

The electric current, I, was recorded under voltage-clamp conditions. The currents were measured by means of a picoamperemeter, digitized by DT2814 (Data Translation, Marlborough, MA) and analyzed by a personal computer. In the majority of experiments the data sampling interval was set at 0.5–5 ms. Ag-AgCl electrodes were put directly into the cell, and a voltage of 65 mV was applied to the BLM.

Photodynamic gramicidin channel inactivation

The photosensitizer, aluminum phthalocyanine bearing from one to four sulfonate groups ($AlPcS_n$, n = 1–4) (Porphyrine Products, Logan, UT), was added to the bathing solution at the *trans* side of the BLM, unless otherwise stated. The phthalocyanines are a complex mixture of isomers because of the possibility of sulfonation at either the 3 or 4 position on the fused phenyl ring and the relative position of sulfonation on adjacent phenyl rings. The $AlPcS_3$, $AlPcS_2$, and $AlPcS_1$ were purified chromatographically to obtain products that contain more than 90% of $AlPcS_n$ with the proper degree of sulfonation, as judged by TLC.

The xenon lamp was placed at the *cis* side of the membrane. Illumination of the bilayer was performed by single flashes (flash energy of ~400 mJ/cm^2 and flash duration <2 ms). The light was passed through a cutoff filter (for wavelengths < 500 nm). In the experiment presented in Fig. 6, a He-Ne laser providing an incident power density of 30 mW/cm^2 was used for excitation. The experiments were carried out at room temperature (T = 20–22°C).

In the presence of the photosensitizer, a light flash is known to decrease the gramicidin-mediated transmembrane current, $I(t)$. The latter is a monoexponential function of time (Rokitskaya et al., 1996):

$$I(t) = (I_0 - I_\infty)e^{-t/\tau} + I_\infty, \quad (1)$$

where I_0, I_∞, and τ are the initial current before illumination, the steady-state current measured after light exposure, and the characteristic time of photoinactivation, respectively. It has been shown that τ is not related to the rate of photoinactivation but rather to the dissociation kinetics of gramicidin dimers (Rokitskaya et al., 1996). Another important parameter is the relative amplitude of photoinactivation, α:

$$\alpha = (I_0 - I_\infty)/I_0. \quad (2)$$

Because the decrease in the gramicidin-mediated current is due to the reduction of the number of open channels, while the single-channel conductance remains unaltered (Rokitskaya et al., 1993), α is equal to the portion of photoinactivated gramicidin channels. This parameter allows us to compare the efficiency of different photosensitizers.

Binding assay

Large unilamellar vesicles (LUVs) were prepared by an extrusion technique (MacDonald et al., 1991), using the small-volume apparatus Liposo-Fast (Avestin, Ottawa, Canada), with filters of 100-nm pore diameter. The final lipid concentration was 0.5 mg DPhPC in 1 ml of a buffer solution containing 1 mM KH_2PO_4 and 20 mM KCl. In some of the experiments 10 mM KCl was replaced by 10 mM NaF. Different amounts of $AlPcS_n$ were added from 10 mM aqueous (n = 2, 3, 4) or 1:1 methanol and 0.1 M NaOH stock solutions ($AlPcS_1$). Upon membrane binding, $AlPcS_n$ introduces some surface charge to the initially uncharged DPhPC bilayer. The surface charge, in turn, gives rise to an electrophoretic mobility, v, of the LUVs. The latter was measured by a zeta-sizer (model DELSA 440 SX; Coulter Electronics, Hialeah, FL) with a four-beam electrophoretic laser scattering analyzer and used to calculate the electrical potential, ζ, at the shear plane. This plane defining what migrates in the electric field is ~0.2–0.4 nm beyond the charged vesicle surface (Carnie and McLaughlin, 1983). The magnitude of the ζ-potential is, thus, less than the surface potential, $\varphi_s(0)$, and it is related to $\varphi_s(0)$ by the Gouy-Chapman theory (see the Appendix).

UV-VIS spectroscopy

$AlPcS_1$ or $AlPcS_4$ (5 μM) was added to liposome suspensions with a final DPhPC concentration of 0.05 mg/ml. The solutions were buffered with 1 mM NaH_2PO_4 at pH 7.0. Samples containing 20 mM KCl or 10 mM NaF and 10 mM KCl were compared with each other. They were analyzed

within 1 h after preparation by a computer-controlled UV-VIS spectrometer (model Lambda 2; Perkin and Elmer, Überlingen, Germany) operated at room temperature (~25°C). The bandwidth was fixed at 0.4 nm. For background correction, the absorption spectrum of a photosensitizer-free liposome suspension was measured simultaneously. The spectra were measured with a 10-mm optical path quartz cuvette.

RESULTS

A typical experimental record that illustrates the flash-induced decrease in the gramicidin-mediated current is shown in Fig. 1. No decrease in the current is observed in the absence of the photosensitizer. The number of photoinactivated gramicidin channels depends on the concentration of phthalocyanines. Up to some maximum value, the parameter α increases with increasing photosensitizer concentration (Fig. 2 *A*). Further additions of $AlPcS_n$ for $n = 1$–3 lead to a decrease in α. As the degree of sulfonation increases, the peak position shifts toward higher concentrations. In this row $AlPcS_1$ is an exception. Fig. 2 *B* shows that the characteristic time (τ) of photoinactivation remains constant in the whole range of $AlPcS_3$ concentrations used. As shown previously (Rokitskaya et al., 1996), τ is close to the gramicidin channel lifetime, an intrinsic channel parameter, and does not depend on the efficiency of interaction with photosensitizer-generated reactive oxygen species.

The decrease in α observed at high concentrations of phthalocyanines can be attributed to quenching of reactive oxygen species (Krasnovsky et al., 1992) and self-quenching of the triplet state of the photosensitizers by their ground state (Foley et al., 1997). The results presented in Fig. 3 support this hypothesis. Actually it is seen that the increase in the concentration of $AlPcS_3$ added at the *trans* side from 1.3 μM to 13 μM leads to the decrease in α regardless of the presence of 1.3 μM $AlPcS_3$ at the *cis* side. To compare the quenching effect of the phthalocyanines with the action of a commonly used quencher of reactive oxygen species, ascorbate (Chou and Khan, 1983; Beyer, 1994), we have tested the influence of the latter on gramicidin channel photoinactivation. As seen from Fig. 4, addition of ascorbate at the *trans* side results in a significant decrease in α. The decrease

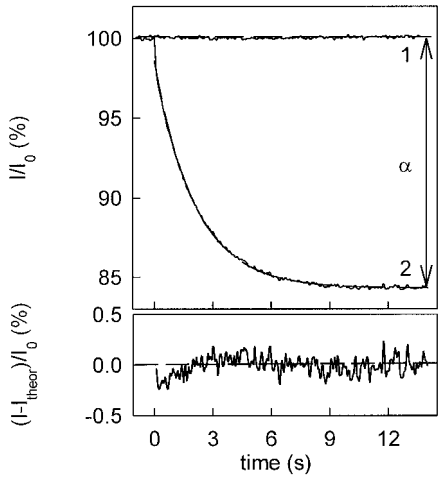

FIGURE 1 The time course of the decrease in the gramicidin-mediated current (I) across the BLM after a flash of visible light (at $t = 0$ s) in the presence of 1.3 μM $AlPcS_3$ (*curve 2*) at the *trans* side of the BLM and the deviation of the data from the monoexponential curve with $\alpha = 16.3\%$, $\tau = 2.1$ s ($I - I_{theor}$). Curve 1 is the time course of the current in the absence of $AlPcS_3$. The normalized values of the current (I/I_0) are plotted versus the time (t). The initial value of the current (I_0) is ~1.0 μA. The dashed curve is the best fit monoexponential curve.

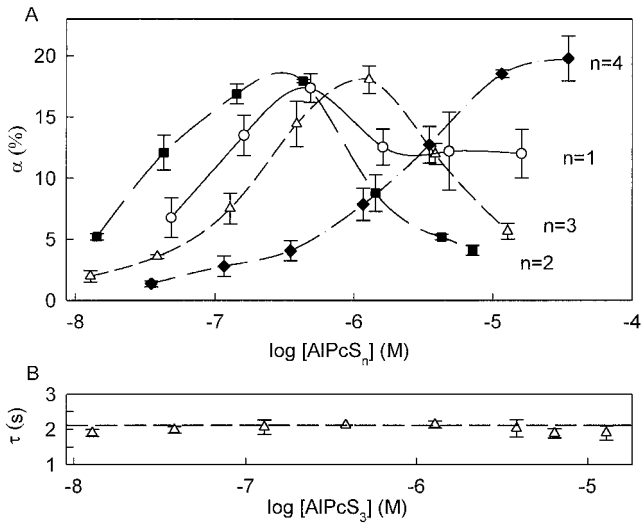

FIGURE 2 (*A*) The dependence of the relative amplitude of gramicidin photoinactivation (α, mean ± SE, %) on the concentration of phthalocyanines with different numbers of sulfonate groups (n). Phthalocyanines with $n = 2$–4 are added from aqueous solutions, while $AlPcS_1$ is dissolved in 1:1 methanol and 0.1 M NaOH. The initial value of the current (I_0) is ~1.0 μA. (*B*) The dependence of the characteristic time of photoinactivation (τ, mean ± SE) on the $AlPcS_3$ concentration.

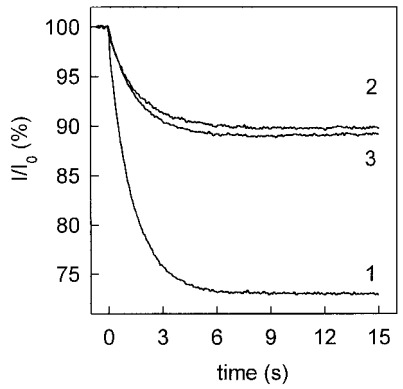

FIGURE 3 The time course of the decrease in the gramicidin-mediated current (I) across BLM after a flash of visible light (at $t = 0$ s) in the presence of 1.3 μM $AlPcS_3$ at the *cis* side of the BLM (*curve 1*), 1.3 μM $AlPcS_3$ at the *cis* side and 13 μM $AlPcS_3$ at the *trans* side of the BLM (*curve 2*), and 13 μM $AlPcS_3$ at the *trans* side of the BLM (*curve 3*). $I_0 = 1.0$ μA.

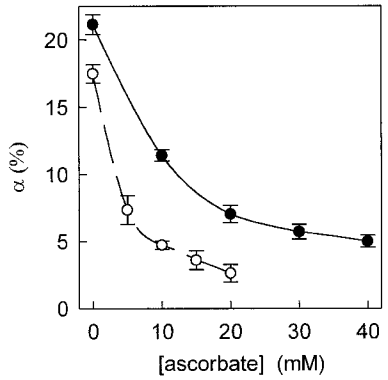

FIGURE 4 The dependence of the relative amplitude of gramicidin photoinactivation (α, mean ± SD, %) on the concentration of ascorbate at the *cis* side (●) and *trans* side (○) of the BLM. $AlPcS_3$ (1.3 μM) is added at the *trans* side of the BLM. I_0 is ~1.5 μA.

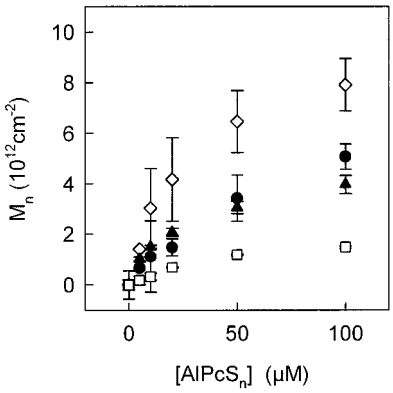

FIGURE 5 The number of photosensitizer molecules, M_n, that are adsorbed to the surface of large unilamellar DPhPC vesicles as a function of photosensitizer concentration. ●, $AlPcS_1$; ◇, $AlPcS_2$; ▲, $AlPcS_3$; □, $AlPcS_4$. M_n was obtained as the ratio of surface charge density, σ, divided by the charge of the photosensitizer molecule ($n \cdot e$, $1 \le n \le 4$; e is electron charge). σ was calculated from the vesicle electrophoretic mobility measured with 20 mM KCl in the buffer solution.

in α is ~50% less pronounced when ascorbate is added at the *cis* side. Taking into account that ascorbate cannot permeate through BLMs (Rose, 1987; Sapper et al., 1985), these data indicate that the mean diffusion distance of the reactive oxygen species in our system is comparable to the BLM width.

Differences in $AlPcS_n$ adsorption to BLM are hypothesized to be responsible for the observed divergence in the photosensitized inactivation of gramicidin channels (Fig. 2). Adsorbing to the neutral vesicle surface, the most hydrophilic phthalocyanine bearing four charged sulfonate groups, induced the smallest changes in the bilayer surface charge density σ. With a decreasing number of sulfonate groups, σ increased. $AlPcS_2$ brought more charges to the bilayer surface than any of the other aluminum phthalocyanines. By dividing the surface charge density at each concentration by the charge per molecule, $n \cdot e$, we obtain the number of bound $AlPcS_n$, M_n. Fig. 5 shows that the disulfonated compound exhibits the highest affinity to the lipid bilayer, followed by the mono-, tri-, and tetrasulfonated phthalocyanine. A comparison with the potency to sensitize gramicidin channel inactivation reveals a qualitative coincidence: it follows the same trend. Obviously, there is a correlation between the affinity for the lipid bilayer and the photosensitizing activity of aluminum phthalocyanines.

The latter is expected to increase significantly after the addition of fluoride, because NaF has been shown to produce an increase in the triplet lifetime, triplet yield, and singlet-oxygen yield (Rosenthal et al., 1994; Bishop et al., 1996). However, it was found in our experiments that fluoride protects gramicidin channels against photoinactivation upon continuous illumination of BLM in the presence of $AlPcS_3$ (Fig. 6). Moreover, the relative amplitude of the flash-induced gramicidin photoinactivation decreased dramatically when NaF and the photosensitizer were present on the same side of the membrane (Fig. 7 *A*). No protective effect was observed when these substances were located at opposite sides of the membrane (Fig. 7 *A*).

Based on the established correlation between the photosensitizing activity and the affinity for the lipid bilayer (Figs. 2 and 5), the protective effect of fluoride (Figs. 6 and 7) is supposed to be due to a decrease in phthalocyanine membrane binding. The validity of this assumption has been confirmed by measurements of the vesicle electrophoretic mobility. In the presence of 10 mM NaF, the ζ-potential induced by the adsorption of $AlPcS_4$, $AlPcS_3$, and $AlPcS_2$ is significantly smaller than in the absence of fluoride anions (Fig. 8). However, with the monosulfonated compound, fluoride produced the opposite effect, i.e., it brought about an increase in membrane binding of $AlPcS_1$ (Fig. 8). As

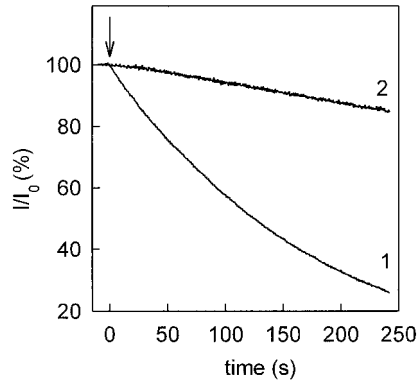

FIGURE 6 The kinetics of the decrease in the gramicidin-mediated current (I) across the BLM induced by illumination with a He-Ne laser (30 mW/cm^2) in the presence of 1.3 μM $AlPcS_3$ at the *trans* side of the BLM (*curve 1*), and 1.3 μM $AlPcS_3$ and 10 mM NaF at the *trans* side of the BLM (*curve 2*). I_0 = 1.0 μA. The illumination is switched on at the moment marked by an arrow.

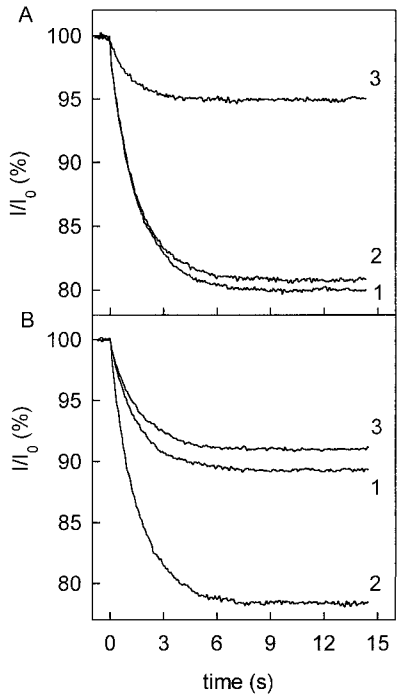

FIGURE 7 (*A*) The time course of the decrease in the gramicidin-mediated current (*I*) across the BLM after a flash of visible light (at $t = 0$ s) in the presence of 1.3 μM $AlPcS_3$ at the *trans* side of the BLM (*curve 1*, $\alpha_1 = 20.2\%$, $\tau_1 = 1.59$ s). *Curve 2*: The addition of 3 mM NaF at the *cis* side of the BLM ($\alpha_2 = 19.1\%$, $\tau_2 = 1.52$ s). *Curve 3*: The addition of 3 mM NaF at the *trans* side of the BLM ($\alpha_3 = 4.0\%$, $\tau_3 = 1.30$ s). (*B*) The time course of the decrease in the gramicidin-mediated current (*I*) across BLM after a flash of visible light (at $t = 0$ s) in the presence of 13 μM $AlPcS_3$ at the *trans* side of the BLM (*curve 1*, $\alpha_1 = 10.3\%$, $\tau_1 = 1.57$ s). *Curve 2*: The addition of 0.2 mM NaF at the *trans* side of the BLM ($\alpha_2 = 21.9\%$, $\tau_2 = 1.56$ s). *Curve 3*: The addition of 3 mM NaF at the *trans* side of the BLM ($\alpha_3 = 9.0\%$, $\tau_3 = 1.60$ s).

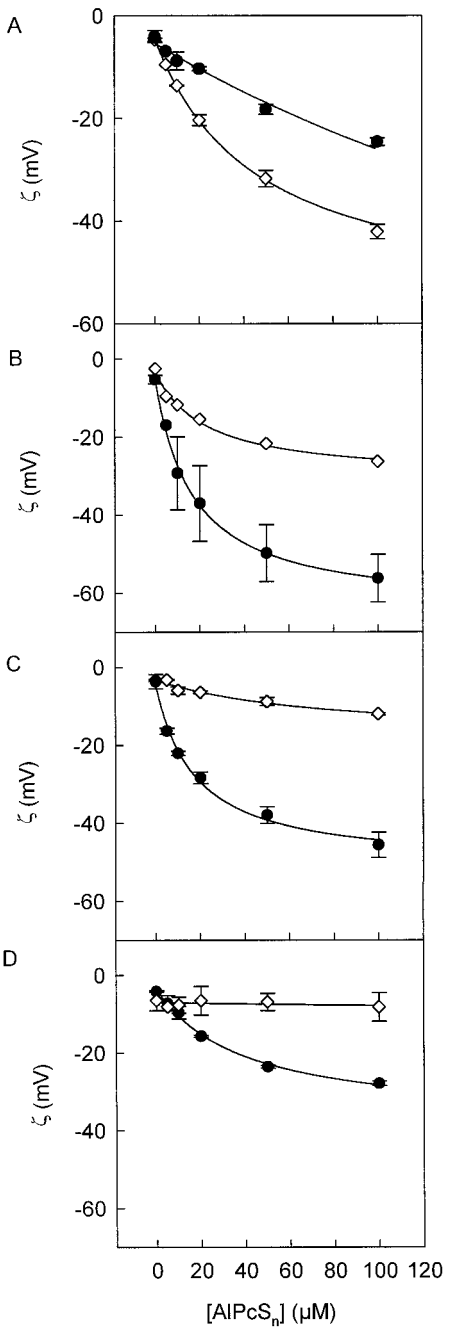

FIGURE 8 The ζ-potential of large unilamellar DPhPC vesicles as a function of photosensitizer (*A*: $AlPcS_1$; *B*: $AlPcS_2$; *C*: $AlPcS_3$; *D*: $AlPcS_4$) concentration in 20 mM KCl (●) or 10 mM KCl and 10 mM NaF (◇) in the buffer solution. The ζ-potential was calculated from vesicle electrophoretic mobility.

seen from Fig. 9, the potency of $AlPcS_1$ in sensitizing gramicidin photoinactivation was reduced by fluoride, although substantially less than with $AlPcS_3$. Here the effect of fluoride can be tentatively explained by a decrease in the dipole strength of the absorption bands in the visible range. In fact, the addition of 10 mM NaF to liposomes containing $AlPcS_1$ led to a dramatic decrease in the Q-band absorption (Fig. 10), in contrast to the data for $AlPcS_4$, where only a blue shift of the peak position without noticeable changes in the dipole strength was observed in the presence of fluoride (Fig. 10). The variations in the effect of fluoride on binding of different sulfonated phthalocyanines to BLM can be associated with the difference in the structure of these compounds in aqueous solutions. It is assumed that $AlPcS_n$ with $n = 2–4$ possesses a hydroxyl axial ligand that is replaced by fluoride in the presence of NaF (Ben-Hur et al., 1992a,c), whereas $AlPcS_1$ possesses a chloride axial ligand (Foley et al., 1997).

From the correlation between the photodynamic activity of aluminum phthalocyanines and their binding to the BLM, an increase in the former may be expected when fluoride is added in the presence of high phthalocyanine concentrations exceeding the optimum (compare Figs. 2 and 3). For low fluoride concentration (0.2 mM) this prediction was found to be true (Figs. 7 *B* and 11). Therefore, partial removal of aluminum phthalocyanine from the membrane surface in-

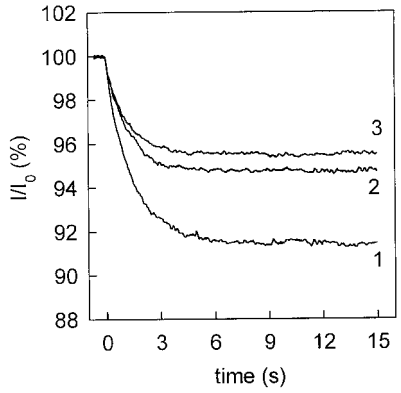

FIGURE 9 The time course of the decrease in the gramicidin-mediated current (I) across the BLM after a flash of visible light (at $t = 0$ s) in the presence of 0.16 μM $AlPcS_1$ at the *trans* side of the BLM (*curve 1*, $\alpha_1 = 8.3\%$, $\tau_1 = 1.43$ s). *Curve 2*: The addition of 3 mM NaF at the *trans* side of the BLM ($\alpha_2 = 5.3\%$, $\tau_2 = 1.22$ s). *Curve 3*: The addition of 10 mM NaF at the *trans* side of the BLM ($\alpha_2 = 4.4\%$, $\tau_2 = 1.14$ s).

duced by fluoride leads to lowering of the photosensitizer concentration at the surface and thereby to a decrease in quenching of reactive oxygen species. At higher concentrations of fluoride, a further decrease in membrane binding of the photosensitizer was induced, and the photoprotective effect prevailed. In contrast to the nonmonotonic dependence of α on the concentration of sodium fluoride that was

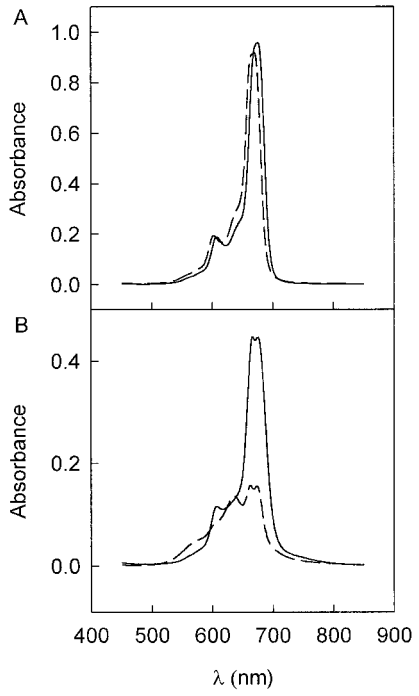

FIGURE 10 The absorption spectra of 5 μM $AlPcS_4$ (*A*) and 5 μM $AlPcS_1$ (*B*) in vesicle suspension that contains 20 mM KCl (*spline line*) or 10 mM KCl and 10 mM NaF (*dashed line*). The final lipid concentration was 0.05 mg/ml.

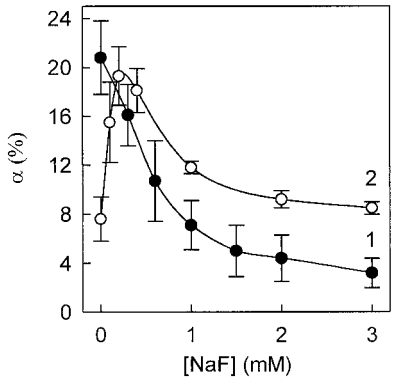

FIGURE 11 The dependence of the relative amplitude of flash-induced photoinactivation (α, mean $\pm$ SD, %) on the concentration of NaF at different concentrations of $AlPcS_3$ (*curve 1*, ●, 1.3 μM; *curve 2*, ○, 13 μM). $AlPcS_3$ and NaF were added at the *trans* side of the BLM. $I_0 = 1.5$ μA.

measured at high concentrations of $AlPcS_3$, the dependence measured at 1.3 μM $AlPcS_3$ did not show a maximum (Fig. 11).

Thus the potency of sensitizing gramicidin inactivation depends on the surface density of adsorbed di-, tri, and tetrasulfonated compounds in the presence as well as in the absence of fluoride anions. A quantitative analysis of this functional relationship requires a knowledge of the respective binding constants. Obviously, they can be obtained only when the concentration, $c(0)$, of the photosensitizer adjacent to the membrane is known. It differs from the bulk concentration, c_b, due to electrostatic repulsion. According to the Boltzmann distribution, the local concentration of any ion is a function of the electric surface potential, $\varphi_s(0)$, and its charge:

$$c(0) = c_b \cdot e^{-z_{eff} e_0 \varphi_s(0)/k_B T}, \quad (3)$$

where e_0, k_B, and T are, respectively, the elementary charge, Boltzmann's constant, and the absolute temperature. Taking into account the finite size of $AlPcS_n$ molecules, the real charge of the photosensitizer is replaced by its effective charge, z_{eff}. This approach is based on the extended Gouy-Chapman theory of the electrostatic diffuse double layer (Alvarez et al., 1983). According to Eq. 3, z_{eff} can be obtained by measurements of $\varphi_s(0)$ at two arbitrarily chosen concentrations of the background electrolyte. Therefore, two $AlPcS_n$ bulk concentrations should be found that give the same surface charge density and thus correspond to the same $c(0)$. In Fig. 12, the dependence of $\varphi_s(0)$ on the $AlPcS_3$ bulk concentration is shown for 10 mM, 20 mM, and 100 mM KCl. The electrophoretic mobility and hence $\varphi_s(0)$ decreased with an increase in the ionic strength. As expected, the electrostatic effects are effectively screened out at high salt concentrations. However, our attempt to calculate z_{eff} from two experiments with different KCl concentrations has failed because of the large variance in the values obtained for z_{eff}, which shows the inadequacy of the ap-

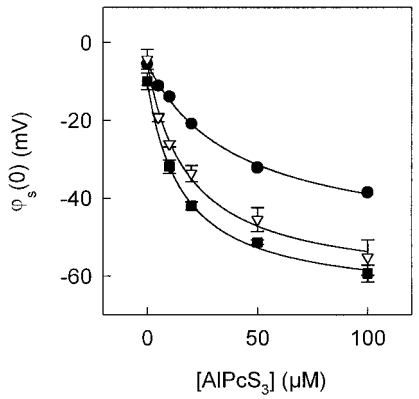

FIGURE 12 Surface potential $\varphi_s(0)$ of large unilamellar DPhPC vesicles as a function of $AlPcS_3$ concentration. With an increase of the ionic strength from 10 mM KCl to 20 mM KCl, and finally to 100 mM (*from the bottom to the top*), the electrophoretic mobility and hence $\varphi_s(0)$ decreased.

proach used. The extended Gouy-Chapman model predicts that the effective charge of a large ion is smaller than its real charge, and that the magnitude of this discrepancy decreases as the ionic strength decreases (Carnie and McLaughlin, 1983).

Assuming that binding to the membrane obeys the Langmuir isotherm, it is possible to calculate the steady-state lipid binding constant (compare, e.g., Pohl et al., 1998) as well as z_{eff} (Denisov et al., 1998) from the dependence of σ on c_b. However, within reasonable limits of both parameters, a satisfactory fit to the data shown in Fig. 5 was not obtained for any of the $AlPcS_n$. Application of the Volmer isotherm, which should be more appropriate because it describes nonlocalized binding of the ligand to the surface, led to the same result. In view of the extremely high concentrations of the photosensitizers used to measure the electrophoretic mobility, we suppose that the formation of aggregates is responsible for the observed deviations from a Langmuir or Volmer isotherm. Although aluminum phthalocyanines do not aggregate in aqueous solutions, they tend to form dimers in organic solvents (Yoon et al., 1993) and are known to form aggregates in cells (Berg et al., 1989a), possibly upon binding to membranes.

DISCUSSION

The concentration dependencies of phthalocyanine-sensitized gramicidin channel photoinactivation and phthalocyanine adsorption on bilayer lipid membranes have revealed a qualitative correlation with each other. The lower the degree of sulfonation, the higher is the binding affinity and the higher is the portion of photoinactivated gramicidin channels. In this row, $AlPcS_1$ is an exception. The concentration at which the relative amplitude of photoinactivation, α, is at half-maximum amounts to 80 nM, 30 nM, 200 nM, and 2 μM for $AlPcS_1$, $AlPcS_2$, $AlPcS_3$, and $AlPcS_4$, respectively. Qualitatively similar dependencies of phototoxicity on the number of sulfonate groups were reported for cells (Paquette et al., 1988; Berg et al., 1989a,b; Chatlani et al., 1991; Ben-Hur et al., 1993; Chan et al., 1990, 1991; Peng et al., 1991).

Similar effects of fluoride ions on sensitized gramicidin inactivation and phthalocyanine binding to membranes provide further evidence for the correlation between photodynamic activity and lipid affinity of photosensitizers. According to the literature (Ben-Hur et al., 1991, 1992b, 1993), fluoride is known to inhibit erythrocyte photohemolysis, spectrin cross-linking, and photoinactivation of viruses sensitized by aluminum phthalocyanines. This inhibition is ascribed to some perturbation of phthalocyanine binding to specific proteins resulting from the formation of aluminum-fluoride complexes. Our data indicate that it is rather the photosensitizer binding to membrane lipids that is required for gramicidin channel photoinactivation to occur. Binding to gramicidin itself is not a prerequisite for photoinactivation, because the negatively charged $AlPcS_3$ is equally efficient in mediating photoinactivation of both neutral gramicidin A and its O-pyromellityl-derivative that bears three negative charges located at the water-membrane interface (Krylov et al., 1998).

The peculiarity of the concentration dependencies of gramicidin inactivation consists in their bell-shaped character. Each of these dependencies has a maximum at a certain concentration that increases with the degree of sulfonation of aluminum phthalocyanine, except for $AlPcS_1$. The decrease in the relative amplitude of gramicidin photoinactivation observed at high phthalocyanine concentrations (Fig. 2) can be explained by:

1. photosensitizer aggregation that results in the reduction of its photosensitizing activity (Berg et al., 1989a);
2. quenching of reactive oxygen species by phthalocyanines themselves (Krasnovsky et al., 1992); and
3. self-quenching of the triplet state of phthalocyanines by their ground state (Foley et al., 1997).

Aggregation is of minor importance because photoinactivation of gramicidin channels sensitized by $AlPcS_3$ adsorbed to only one membrane/water interface is markedly diminished by high surface concentrations of $AlPcS_3$ adsorbed to the opposite membrane/water interface (Fig. 3). From the comparison with the effect of ascorbate (Fig. 4), quenching of reactive oxygen species seems to be a likely mechanism. Our data indicate that the membrane-bound aluminum phthalocyanine molecules preferentially act as quenchers. This conclusion is drawn on the basis of the stimulating effect of fluoride anions on gramicidin photoinactivation observed at high $AlPcS_3$ concentrations (Figs. 7 *B* and 11). The stimulation is ascribed to the release of photosensitizer molecules from the membrane surface. The dependency of the quenching efficiency of aluminum phthalocyanines on their binding to membranes is supported by the fact that $AlPcS_4$, having the lowest membrane affinity,

does not display any decrease in the relative amplitude of gramicidin photoinactivation, even at very high concentrations (Fig. 2).

It should be noted that quenching of reactive oxygen species alone cannot explain the decrease in α observed at high phthalocyanine concentrations. It is known (Foley et al., 1997; Krasnovsky et al., 1992) that both the rate of reactive oxygen species generation and the rate of their quenching depend linearly on the concentration of a photosensitizer if the latter acts also as a reactive oxygen species quencher. On the other hand, it is evident that the decrease in α can occur only if some bimolecular quenching reaction takes place. It is reasonable to suppose this reaction to be self-quenching of the photosensitizer triplet state by its ground state (Foley et al., 1997).

Summarizing, the affinity for membrane lipids is shown to be one of the factors determining the photodynamic activity of aluminum phthalocyanines. In line with the literature data (Kochevar et al., 1994), it appears that the association of the photosensitizer with the lipid bilayer may be more important than its reactive oxygen quantum yield. The effect of fluoride presents a vivid example: despite the increase in the singlet oxygen yield (Rosenthal et al., 1994; Bishop et al., 1996), the photosensitizing efficacy of $AlPcS_n$ decreases because of the reduction in membrane binding.

APPENDIX

By using the Helmholtz-Smoluchowski equation, we calculated the ζ-potential from the measured electrophoretic mobility, v:

$$\zeta = \frac{\eta v}{\epsilon_0 \epsilon_r E}, \tag{4}$$

where ϵ_r, ϵ_0, η, and E are the dielectric constant of the aqueous solution, the permittivity of free space, the viscosity of the aqueous solution, and the electric field strength, respectively. Subsequently, the Gouy-Chapman theory was applied to obtain the surface potential $\varphi_s(0)$ from $\zeta\,(\varphi_s(x) = \zeta$, with $x = 0.2$ or 0.4 nm at 100 mM or 20 mM KCl (Carnie and McLaughlin, 1983), respectively):

$$\varphi_s(x) = \frac{2k_BT}{ze_0} \ln\left[\frac{1 + \beta \cdot e^{-\kappa \cdot x}}{1 - \beta \cdot e^{-\kappa \cdot x}}\right], \tag{5}$$

with

$$\beta = \frac{e^{\Psi} - 1}{e^{\Psi} + 1}, \quad \Psi = \frac{ze_0\varphi(0)}{2k_BT}, \quad \kappa = \sqrt{\frac{2e^2z^2N_AJ}{\epsilon_0\epsilon_rk_BT}}, \tag{6}$$

where x, k, N_A, T, and J denote the distance to the membrane, the Boltzmann constant, the absolute temperature, and the ionic strength of the solution, respectively. It is assumed that the contribution of the negatively charged photosensitizer molecules is negligible because their bulk concentration is at least two orders of magnitude smaller than the bulk KCl concentration, and because of electrostatic repulsion, the concentration difference in the immediate membrane vicinity is even bigger.

$\varphi_s(0)$ is related to the surface charge density, σ, of the membrane:

$$\frac{\sigma}{\sqrt{8\epsilon_0\epsilon_rN_Ak_BTJ}} = \sinh\left[\frac{e_0\varphi_s(0)}{2k_BT}\right]. \tag{7}$$

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