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Interaction of Tetrasubstituted Cationic Aluminum Phthalocyanine with Artificial and Natural Membranes

A. A. Pashkovskaya[1], I. V. Perevoshchikova[1], V. E. Maizlish[2], G. P. Shaposhnikov[2], E. A. Kotova[1], and Yu. N. Antonenko[1]*

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: antonen@genebee.msu.ru*
[2]*Ivanovo State University of Chemistry and Technology, pr. F. Engelsa 7, 153000 Ivanovo, Russia*

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Abstract—A study of the properties of water-soluble tetrasubstituted cationic aluminum phthalocyanine ($AlPcN_4$) revealed efficient binding of this photosensitizer to phospholipid membranes as compared with tetrasulfonated aluminum and zinc phthalocyanine complexes. This also manifested itself in enhanced photodynamic activity of $AlPcN_4$ as measured by the photosensitized damage of gramicidin channels in a planar bilayer lipid membrane. The largest difference in the photodynamic activity of cationic and anionic phthalocyanines was observed in a membrane containing negatively charged lipids, thereby pointing to significant contribution of electrostatic interactions to the binding of photosensitizers to a membrane. Fluoride anions suppressed the photodynamic activity and binding to membrane of both tetraanionic and tetracationic aluminum phthalocyanines, which supports our hypothesis that interaction of charged metallophthalocyanines with phospholipid membranes is mostly determined by coordination of the central metal atom with the phosphate group of lipid.

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Key words: planar bilayer lipid membrane, photosensitizer, phthalocyanine, singlet oxygen, ion channels, gramicidin A, liposomes, mitochondria

Due to their unique properties, phthalocyanines are widely used in the design of new materials such as stable dyes, catalysts, building blocks for nanostructures, active components of optical sensors, semiconductor and electrochromic devices, memory systems, liquid-crystal color displays, photoelectric transformers of solar energy, etc. [1-5]. During the last twenty years, phthalocyanines have also been used as photosensitizers in photodynamic therapy of cancer [6] — aluminum, zinc, and silicon phthalocyanines with high quantum yield of singlet oxygen being among the most efficient [7]. Water solubility of some phthalocyanine derivatives resulting from insertion of cationic or anionic substituents is a property important for medical purposes. Positively charged phthalocyanine derivatives [8] appeared to be effective agents for photodynamic inactivation of bacteria [9-14]: their negatively charged envelopes hinder the penetration of anionic photosensitizers. According to numerous data, membranes are the main targets for photodynamic action [15, 16]; that is why clarification of the mechanism of interaction between phthalocyanines and membrane is of great importance. It is known that hydrophobic interactions play an important role in the binding of porphyrins and analogous compounds to lipid membranes [17, 18]. The inhibitory effect of fluoride anions on photodynamic activity and binding of sulfonated aluminum phthalocyanines to membrane shown earlier [19-22] was rationalized on the assumption that hydrophobic interactions play an important role, decreasing binding to membranes due to the increased negative charge of a phthalocyanine molecule bound with a fluorine anion [23]. However, later we obtained data indicating that binding of sulfonated aluminum and zinc phthalocyanines with phospholipid membrane is mostly determined by coordination of the central metal atom with the phosphate group of lipid [24].

Abbreviations: $AlPcN_4$, tetra-tributylammonium salt of hydroxyaluminum tetra-4-chloromethylphthalocyanine; $AlPcS_4$, tetrasulfonated aluminum phthalocyanine; BLM, bilayer lipid membrane; DMFA, N,N′-dimethylformamide; DPhPC, diphytanoyl phosphatidylcholine; DPhPG, diphytanoyl phosphatidylglycerol; $ZnPcS_4$, tetrasulfonated zinc phthalocyanine.

* To whom correspondence should be addressed.

The goal of the present work was to study the photosensitizing activity and binding with membranes of a new cationic aluminum phthalocyanine.

MATERIALS AND METHODS

The tetrabutylammonium salt of hydroxyaluminum tetra-4-chloromethylphthalocyanine ($AlPcN_4$) was obtained by the interaction of hydroxyaluminum tetra-4-chloromethylphthalocyanine with tributylamine according to the following reaction scheme:

$$HOAlPc(4\text{-}CH_2Cl)_4 + N(C_4H_9)_3 \rightarrow HOAlPc(4\text{-}CH_2\text{-}N^+(C_4H_9)_3Cl^-)_4.$$

Hydroxyaluminum tetra-4-chloromethylphthalocyanine (150 mg, 0.2 mmol) was dissolved in N,N′-dimethylformamide (DMFA) (6 ml) and 5% ethanolic solution of tributylamine (24.2 ml) was added. The reaction mixture was heated to boiling and thus kept for 7 h. After cooling, the reaction mixture was poured into water (50 ml) and filtered. The filtrate was evaporated under vacuum, and the product was washed with dimethyl ether and dried. Yield 190 mg (63%). Found (%): C 68.53, H 9.27, N 10.99, Cl 8.71. Calculated for $C_{84}N_{12}H_{129}Cl_4AlO$ (%): C 67.63, H 8.72, N 11.27, Cl 9.51.

Tetrasulfonated aluminum ($AlPcS_4$) and zinc ($ZnPcS_4$) phthalocyanines were obtained from Porphyrin Products (USA). Gramicidin A was from Sigma (USA) and lipids from Avanti Polar Lipids Inc. (USA). Planar bilayer lipid membrane was formed from 2% decane solution of lipid according to Mueller [25] (holes 0.55 mm in diameter in a Teflon septum separating two water phases containing 100 mM KCl, 10 mM MES, 10 mM Tris, 10 mM β-alanine, pH 7). In most cases, peptide solutions at various concentrations were added at the *cis*-side of the membrane. By convention, the side of the cell at which positive potential is applied and at which the membrane is exposed to light is called the *cis*-side. Electric current through the membrane was measured using a Keithley 428 amplifier (USA). The analog signal was transmitted to a computer using a LabPC 1200 plate from National Instruments Inc. (USA) and was analyzed using WinWCP Strathclyde Electrophysiology Software by J. Dempster from the University of Strathclyde (England). For recording the membrane conductivity, electrodes made from chlorinated silver foil were immersed directly in the cell. To illuminate the membrane with constant light, an arc lamp with light power 400 mW/cm^2 near the membrane was used. For light flash, a xenon flash lamp with energy 0.3 J and flash duration <3 msec was used.

Electrophoretic mobility of liposomes was measured as described in [22] using a Zetasizer Nano device from Malvern (England). Monolamellar liposomes from egg yolk phosphatidylcholine or *Escherichia coli* total lipids were prepared in solution containing 10 mM KCl, 5 mM MES, and 5 mM Tris, pH 7. Thus obtained multilamellar mixture was filtered through a Nucleopore polycarbonate filter with 0.1 μm holes using an extruder from Avanti Polar Lipids Inc.

Fluorescence spectra of dyes were recorded using a Panorama Fluorat-02 spectrofluorimeter from Lumex (Russia). To determine the quantum yield of singlet oxygen generation of phthalocyanines, kinetics of light-dependent quenching of fluorescence of 9,10-dimethylanthracene (excitation wavelength 375 nm, emission maximum 426 nm) by the action of light with $\lambda = 680$ nm in the presence of metallophthalocyanines according to [26-28] was studied. For $ZnPcS_4$ in DMSO, the quantum yield was taken as 0.68 according to [29].

Mitochondria from rat liver were isolated by differential centrifugation according to a standard procedure. The isolation medium contained 300 mM sucrose, 10 mM MOPS, 1 mM EDTA, pH 7.4. All measurements were performed in buffer containing 10 mM KCl, 70 mM sucrose, 190 mM mannitol, 20 mM HEPES, 5 mM succinate, 0.5 mM EDTA, pH 7.5. Mitochondria concentration in the cuvette was ~0.2 mg/ml.

For fluorescence excitation, a He-Ne laser with $\lambda = 633$ nm on an Olympus IMT-2 epifluorescence inverted microscope (USA) with water-immersion objective 40×, NA from Carl Zeiss Jena (Germany) was used. Fluorescence signal passed through the appropriate dichroic beam splitter and was projected on a 50 μm light guide connected with an SPCM-AQR-13-FC avalanche photodiode from Perkin Elmer Optoelectronics, Vaudreuil (Canada). The signal was transformed using a Flex02-01D/C interface card from Correlator.com (USA) and recorded for 30 sec. The fluorescence was collected from the confocal volume at the distance 50 μm under a thin glass on which 60 μl of mitochondria suspension in buffer was applied.

RESULTS AND DISCUSSION

To evaluate efficiency of photosensitizers, earlier we developed a method of registration of photodamage of the channel-forming peptide gramicidin A based on measurements of decrease in gramicidin-induced current through a model bilayer lipid membrane (BLM) as a response to the action of visible light in the presence of the photosensitizer [22, 30, 31]. Using this method, in this work we studied and compared the photosensitizing activity of tetra-tributylammonium salt of hydroxyaluminum tetra-4-chloromethylphthalocyanine ($AlPcN_4$) and tetrasulfonated aluminum ($AlPcS_4$) and zinc ($ZnPcS_4$) phthalocyanines.

Amplitudes of gramicidin photoinactivation accounting for the fraction of damaged gramicidin channels versus concentration of various phthalocyanines are presented in Fig. 1a. From this figure, it is evident that

tetracationic aluminum phthalocyanine ($AlPcN_4$) exhibits the maximal photodynamic activity in this system compared with the other photosensitizers studied. Activities differ to a greater extent if the negatively charged membrane containing anionic lipids (Fig. 1b) is used instead of membrane of the neutral lipid (diphytanoyl phosphatidylcholine) (Fig. 1a).

The data on photodynamic activity of charged phthalocyanines correlate with liposome ζ-potentials obtained by measuring electrophoretic activity. The data indicate that of all the studied photosensitizers, $AlPcN_4$ most efficiently binds with the negatively charged liposomes from the total *E. coli* lipids (Fig. 2a) as well as with the neutral liposomes from egg yolk phosphatidylcholine (Fig. 2b). Measurements of the quantum yield of singlet oxygen generation φ_Δ using dimethylanthracene as a trap (Fig. 3 and table) showed that for tetracationic aluminum phthalocyanine dissolved in DMSO, φ_Δ is somewhat less than that for $ZnPcS_4$. Thus, significant difference in photodynamic activities of the positively and negatively charged phthalocyanines on lipid membranes, especially on those containing anionic lipids, can be rationalized by the difference in the membrane binding of the phthalocyanines. The data indicate that the role of electrostatic interactions in the binding of charged phthalocyanines with membrane is of considerable importance.

From Fig. 4 we note that KF suppresses photoinactivation of gramicidin channels in the neutral membranes in the presence of both $AlPcN_4$ and $AlPcS_4$ and does not affect photodynamic activity of $ZnPcS_4$, which is in accord with [24]. Fluoride anions inhibit binding of both $AlPcN_4$ and $AlPcS_4$ with membrane (Fig. 5) and negligibly increase φ_Δ for $AlPcN_4$ (table). In the experiments presented in Fig. 5, binding of phthalocyanines with mem-

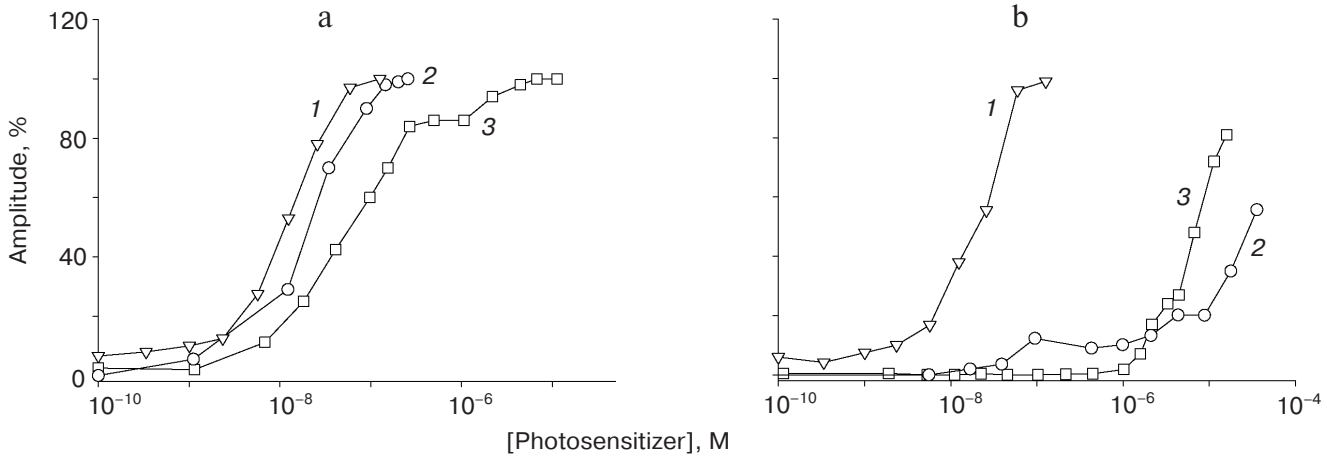

Fig. 1. Amplitudes of gramicidin A photoinactivation versus $AlPcN_4$ (*1*), $ZnPcS_4$ (*2*), and $AlPcS_4$ (*3*) concentrations on membranes of DPhPC (a) and DPhPC/DPhPG (70 : 30%) mixture (b). Buffer contained 100 mM KCl, 10 mM MES, 10 mM Tris, pH 7.

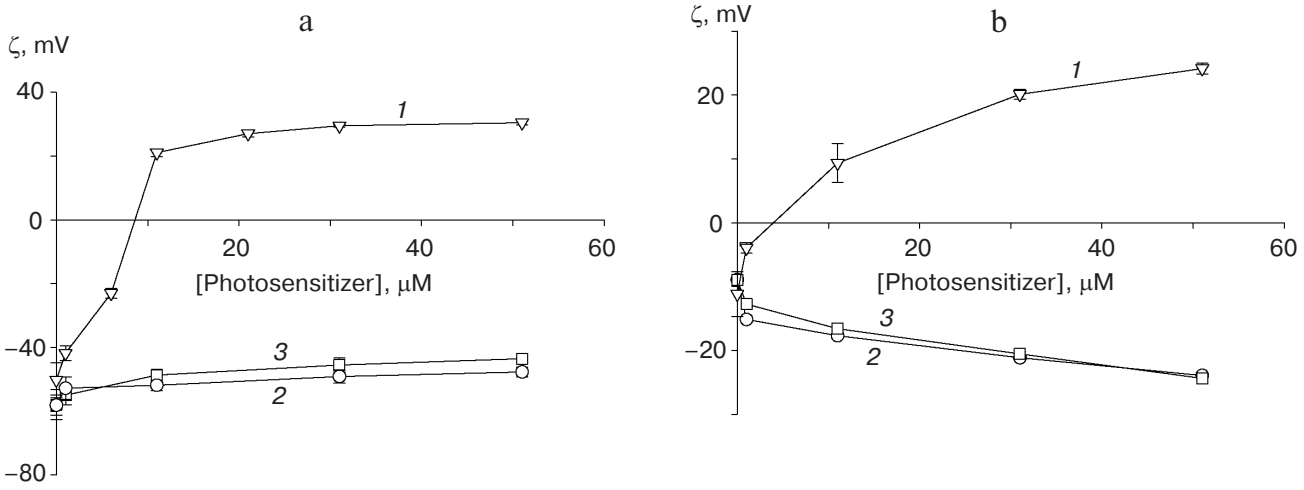

Fig. 2. ζ-Potentials of liposomes from *E. coli* lipids (a) and egg yolk phosphatidylcholine (b) versus $AlPcN_4$ (*1*), $ZnPcS_4$ (*2*), and $AlPcS_4$ (*3*) concentrations. Buffer contained 10 mM KCl, 5 mM MES, 5 mM Tris, pH 7.

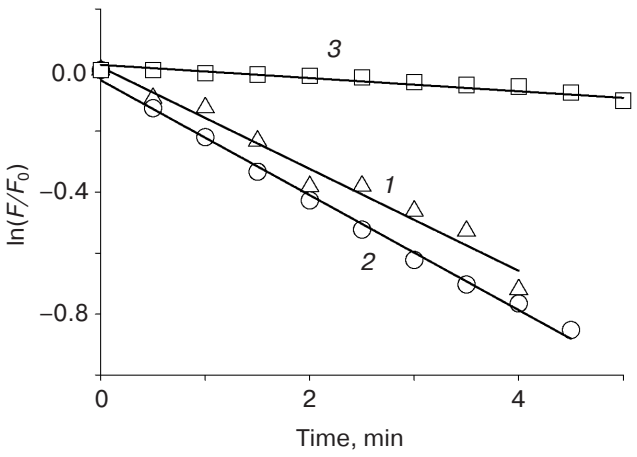

Fig. 3. Kinetics of quenching of fluorescence of 9,10-dimethylanthracene (*F*) in DMSO (λ_{ex} = 357 nm, λ_{em} = 426 nm) by the action of light with λ = 680 nm in the presence of $AlPcN_4$ (*1*), $ZnPcS_4$ (*2*), and $AlPcS_4$ (*3*) relative to the initial fluorescence (F_0).

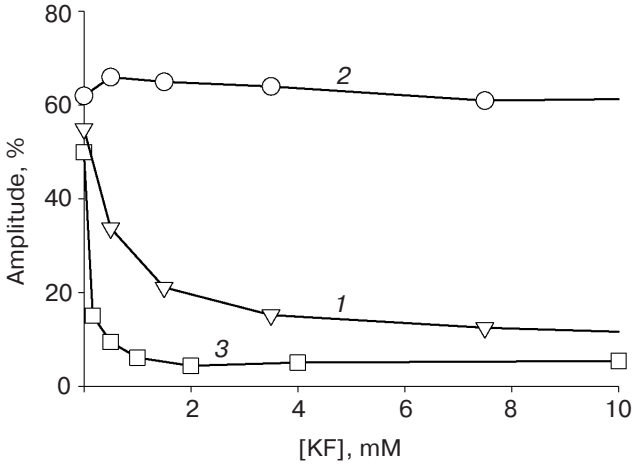

Fig. 4. Amplitude of gramicidin A photoinactivation on DPhPC membrane in the presence of $AlPcN_4$ (*1*), $ZnPcS_4$ (*2*), and $AlPcS_4$ (*3*) versus fluoride anion concentration. Buffer contained 100 mM KCl, 10 mM MES, 10 mM Tris, pH 7.

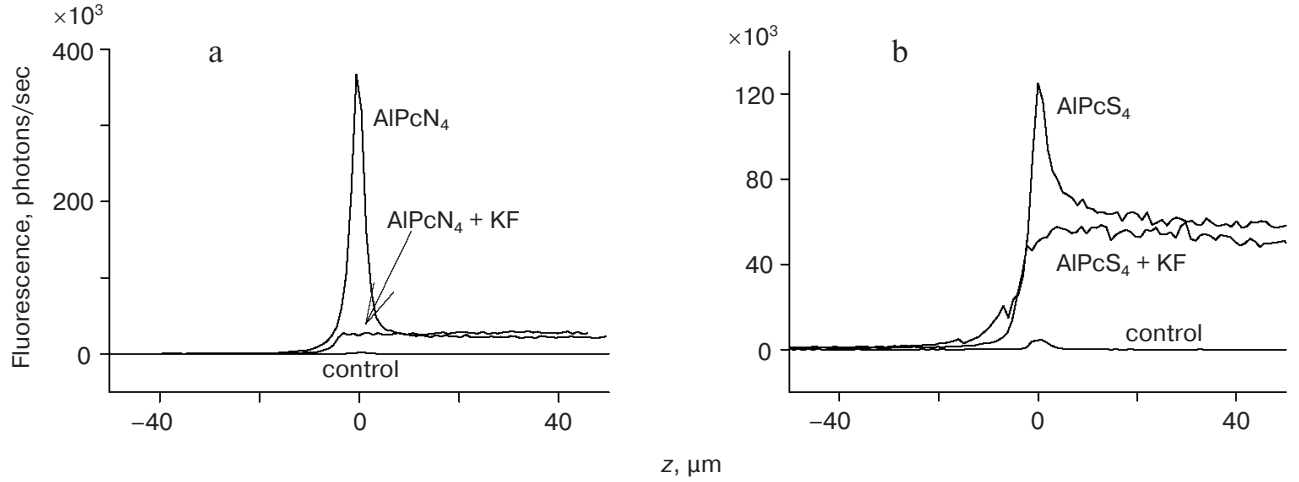

Fig. 5. Height profile of fluorescence signal (*z*-scan) near position of a plane horizontal phospholipid membrane on addition of 0.2 μM $AlPcN_4$ (a) and 0.03 μM $AlPcS_4$ (b) in the absence and in the presence of 10 mM KF. Measurements were performed using a LSM 510 confocal microscope from Carl Zeiss Jena, λ_{ex} = 633 nm. The data were obtained by scanning fluorescence signal by height (*z*) in a special cell allowing formation of a horizontal planar phospholipid membrane. The latter was positioned at *z* = 0 μm. Membrane was formed on a hole in thin Teflon film, which separated the upper compartment containing a dye and the lower compartment containing only buffer solution (100 mM KCl, 10 mM MES, 10 mM Tris, pH 7).

brane was measured via change in fluorescence signal of a microscope operating in confocal mode. This mode was provided by focusing a red laser beam (633 nm) and registration of a part of the fluorescence signal from the area of the maximal excitation intensity. From Fig. 5 we also note that $AlPcN_4$ binding is much more efficient than $AlPcS_4$ binding even in case of the neutral membrane.

We detected a blue shift of the maximum in the fluorescence spectrum of $AlPcN_4$ in ethanol solution caused by the action of fluoride anions (Fig. 6), which was analogous to that of $AlPcS_4$ found earlier [24]. This indicates formation of aluminum phthalocyanine–fluoride complex. Thus, the effect of fluoride is well rationalized if we suggest that $AlPcN_4$ binding with phospholipid membrane is mostly determined by coordination of the central metal atom with the phosphate group of a lipid as in the case of sulfonated metallophthalocyanines [24]. The phosphate group of the lipid might be displaced from the aluminum coordination sphere due to competitive substitution by fluoride anion.

We also studied binding of cationic and anionic phthalocyanines with membranes of isolated mitochon-

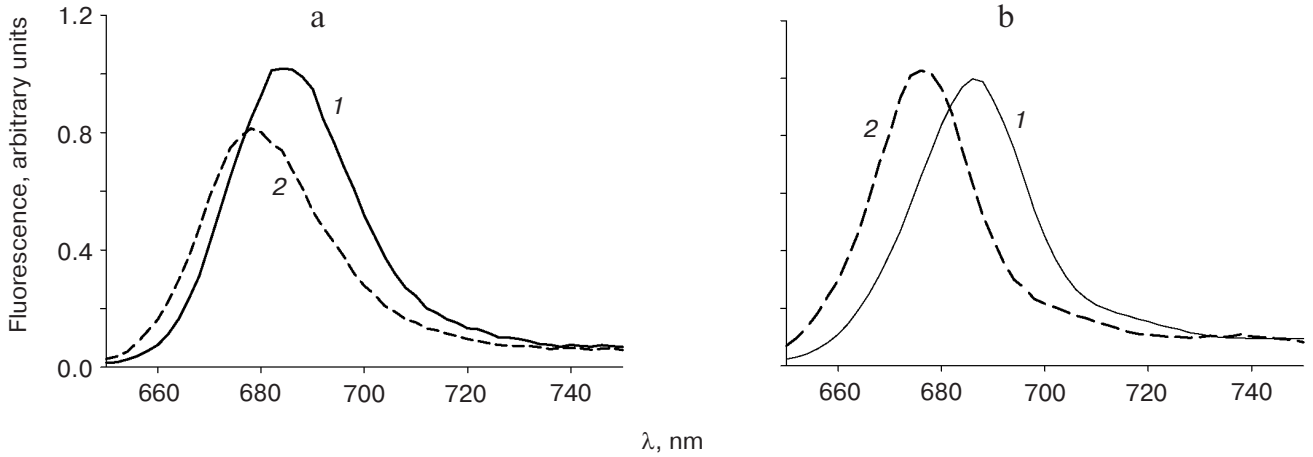

Fig. 6. Fluorescence spectra of $AlPcN_4$ (a) and $AlPcS_4$ (b) in ethanol in the absence (*1*) and in the presence of 1 mM KF (*2*). Excitation wavelength, 400 nm.

dria. Mitochondrial membrane is known to be negatively charged, and this charge is mainly due to the presence of a significant amount of protein in it, e.g. the internal mitochondrial membrane contains up to 80% protein. Binding was evaluated via decrease in absorption of dye solution after centrifugation of a mitochondrial suspension. The positively charged $AlPcN_4$ was found to bind more strongly with mitochondria than the negatively charged phthalocyanines do (Fig. 7).

Binding of $AlPcN_4$ with isolated mitochondria was also studied via signal change of the fluorescence microscope operating in confocal mode. As presented in Fig. 8a, addition of mitochondria to $AlPcN_4$ solution yields fluorescence peaks, which is a typical response on dye binding in such system [32]. Addition of KF resulted in decrease in amplitude and number of fluorescence peaks. Therefore, fluoride anions decrease $AlPcN_4$ sorption also in case of mitochondrial membranes, which can be caused by suppressed $AlPcN_4$ binding both with proteins and lipids. In analogous experiment with $AlPcS_4$ (Fig. 8b), addition of mitochondria did not cause appearance of fluorescence peaks. These data indicate that the latter dye is not bound.

Quantum yield of singlet oxygen generation (φ_Δ) on excitation of metallophthalocyanines

Photosensitizer	φ_Δ
$ZnPcS_4$	0.68 ± 0.03
$AlPcN_4$	0.60 ± 0.10
$AlPcN_4$ + KF	0.63 ± 0.05
$AlPcS_4$	0.10 ± 0.02

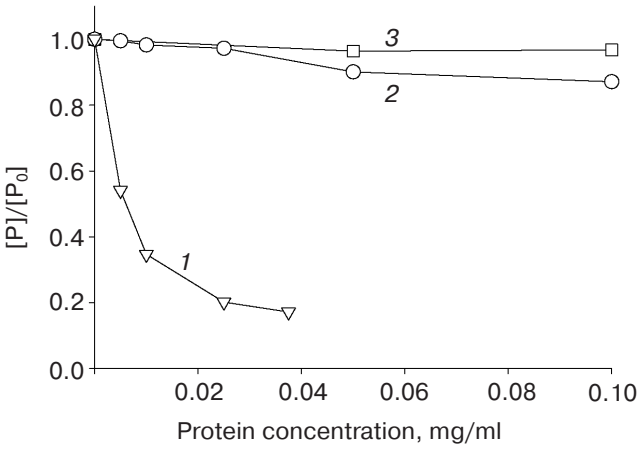

Fig. 7. Ratio of unbound dye concentration (P) to the total dye concentration (P_0) versus mitochondrial protein concentration for $AlPcN_4$ (*1*), $ZnPcS_4$ (*2*), and $AlPcS_4$ (*3*). Buffer contained 250 mM sucrose, 10 mM MES, 10 mM Tris, pH 7.

So, the positively charged aluminum phthalocyanine $AlPcN_4$ having four cationic groups as substituents binds with artificial phospholipid membranes as well as natural mitochondrial membranes more effectively than the negatively charged tetrasulfonated metallophthalocyanines. Therefore, electrostatic interactions play an important role in binding of the charged metallophthalocyanines with membranes.

Efficient binding with membranes together with high quantum yield of singlet oxygen generation provides high photodynamic activity of $AlPcN_4$. In this work, we measured photosensitized inactivation of gramicidin A channels in a BLM as a manifestation of photodynamic activity. It was found that photoinactivation of channels in the presence of $AlPcN_4$ as well as binding of this photosensitizer with membranes is suppressed by fluoride anions. This

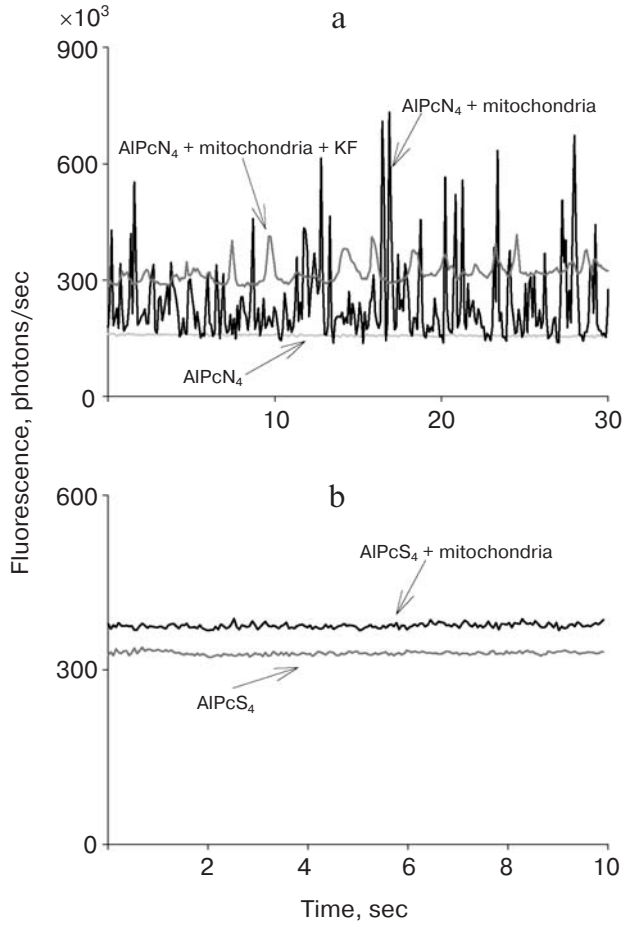

Fig. 8. Fluorescence microscope signal in the presence of metallophthalocyanines and mitochondria. Concentrations: a) 1 μM $AlPcN_4$, 10 mM KF, mitochondria (0.1 mg protein/ml); b) 1 μM $AlPcS_4$, 10 mM KF, mitochondria (0.1 mg protein/ml). Buffer contained 250 mM sucrose, 20 mM MOPS, 1 mM EGTA, pH 7.4.

might be caused by coordination of fluoride anion by the central aluminum atom, which hinders aluminum coordination by the phosphate groups of phospholipids or membrane proteins (in the case of mitochondrial membranes).

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