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A single neuron response to photodynamic effect of various aluminum and zinc phthalocyanines

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Abstract

The photodynamic effects of sulphonated zinc and aluminum phthalocyanine derivatives as well as phosphonated aluminum phthalOcyanine on the firing of isolated crayfish mechanoreceptor neurons were studied. After 30 min staining neurons were irradiated with He-Ne laser (632.8 nm, 0.3 W/cm^2) and changes in neuron firing frequency were recorded. Neuron firing was found to be very sensitive to photodynamic effect and could serve as a sensitive indicator of cell photodamage. It changed the firing level and then died at nanomolar concentrations of phthalocyanines. The dynamics of the neuron responses to photodynamic effects included stages of firing activation and/or inhibition prior to irreversible firing abolition. The order of these stages depended on photosensitizer type and concentration. The comparison of the dependencies of neuron lifetime on photosensitizer concentrations showed $ZnPcS_2$ to be the most effective photosensitizer.

Keywords: Photodynamic effect; Zn phthalocyanines; Al phthalocyanines; Neuron; Photosensitizers

Introduction

Photodynamic (PD) therapy is a promising cancer treatment modality. It includes localized delivery of laser light to the stained tissue and photoexcitation of photosensitizer (PS) molecules. The latter generate cytotoxic free radical products and singlet oxygen ($^1O_2$) selectively killing tumor cells. Phthalocyanines are receiving increasing attention as second-generation photosensitizers for PD therapy. Sulphonated Al and Zn phthalocyanines demonstrate favorable physico-chemical and spectral properties for the use as photosensitizers. Some of them are undergoing clinical trials [1–4]. Phosphonated aluminum phthalocyanine has been also demonstrated to be an effective PS [5]. PD cell killing is used not only in clini-

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cal medicine but serves also as an experimental tool in neurophysiological experiments. For example, selective photoinactivation of the stained neurons has been used for the mapping of simple nervous systems and study of the role of the certain neurons in animal behavior [6,7].

However, the dynamics and mechanisms of nerve cell responses to PD effect are nor sufficiently studied. Recently an isolated crayfish mechanoreceptor neuron (non-traditional for PD researches but informative model object) has been proposed to use in the study of PD effect at the cellular level and comparison of different photosensitizers [8,9]. Biochemistry, physiology, ultrastructure, and photobiological responses of this classic neurophysiological object are well studied [10–12]. Its attractive advantage is the ability to fire with a nearly constant rate during several hours. This stable background provides the continuous recording of the cell response dynamics from initial threshold shifts to terminal events leading to the cell death. In our preliminary studies this cell was demonstrated to be very sensitive to PD effect [8,9].

The aim of the present work was to study the dynamics of neuron response to PD effect of different aluminum and zinc phthalocyanine derivatives in order to compare their PD efficiencies and study some mechanisms of PD effect of these PSs at the cellular level.

Methods

Slowly adapting muscle receptor organ of the crayfish *Astacus leptodactilus* was isolated as described by Wiersma et al. [13] and placed into a plexiglass chamber with van Harreveld saline (mM: NaCl - 205; KCL -5.4; $NaHCO_3$ - 0.24; $MgCl_2$ - 5.4; $CaCl_2$ - 13.5; pH 7.2–7.4). In this preparation, stretch receptor neurons could fire 8–12 hours at a nearly constant rate. Spikes were derived extracellularly by the suction electrode from axon and amplified (the amplifier UU-90, IEM, Leningrad, Russia). Their frequency was converted into voltage by the analog frequency meter MFU-1 (IEM, Leningrad, Russia) and continuously recorded by the chart-recorder N-390 (ZIP, Krasnodar, USSR). To test the irreversibility of neuron activity abolition we recorded potentials 30–60 min after spike cessation and then adequately stimulated mechanoreceptor neuron by the application of additional receptor muscle extension. The absence of spikes indicated that neuron had lost the ability to fire. Such irreversible abolition of firing was considered as a functional hallmark of the neuron death. Neuron lifetime was measured from the irradiation start to the firing abolition moment.

At the beginning of each experiment the initial neuron frequency was set near 10–15 Hz by application of the appropriate receptor muscle extension. After 30 min ‘control’ recording of spike generation, the PS solution was added into the experimental chamber. After further 30 min, cells were irradiated with the helium-neon laser (632.8 nm, 0.3 W/cm^2, LGN-111, «Polyaron», Lvov, USSR,) until the irreversible firing cessation. The irradiation power was measured by the laser dosimeter (IMO-2N, «Etalon», Volgograd, USSR). The irradiation exposures were as long as the neuron lifetimes.

The following photosensitizers synthesized in NIOPIK, Moscow, Russia were studied:

- Photosens: sodium salt of sulphonated aluminum phthalocyanine, $AlPcS_n$. This is a mixture of compounds with different sulphonation degrees: $n = 2$, 3, or 4. The mean $n \approx 3.1$.
- Sodium salts of di-, tri- and tetrasulphonated zinc phthalocyanines: $ZnPcS_n$. These are also the complex mixtures with the mean $n \approx 2.13$, 3.06 and 3.74, respectively ($ZnPcS_2$, $ZnPcS_3$ and $ZnPcS_4$).

- Phosphonated aluminum phthalocyanine (5): octa(oxyetoxyphosphinylmethyl) phthalocyanine aluminum hydroxide HOAlPc $[PO(OC_2H_5)OH]_8$, or PAlPc.

Their formulae, composition and properties are illustrated in Fig. 1 and Table 1.

All experiments were carried out at a room temperature of 20±3 °C. Standard statistical methods including correlation and regression analysis [12] were used. To compare efficiencies of different photosensitizers we studied concentration dependencies of neuron lifetime *(T)*. Functions *T(C)* were approximated by the power functions: $T(C) = a^* C^b$, which are linear in the double logarithmic coordinates: $lg\ T = lg\ a + b^*\ lg\ C$. Parameters *a*, and *b* were determined by the least-squares method.

Results

The dynamics of the neuron responses to photodynamic effect

As in our previous papers [8,9], the frequency of isolated neuron firing was found to be insensitive to either He-Ne laser irradiation (632.8 nm), or to staining alone. However, it was extremely sensitive to the combined action of these factors, i.e. to PD effect. Neuron response dynamics included alternating phases of firing acceleration and/or inhibition depending on PS type and concentration. The main observed types of neuron response dynamics were (Fig. 2): E - firing activation followed by abrupt irreversible firing abolition; EIE - activation, inhibition, and new activation followed by abrupt firing cessation; IE - initial transient inhibition and then firing activation followed by abrupt firing abolition; I - gradual firing inhibition until irreversible cessation; EI - transient activation of firing and then gradual inhibition until irre-

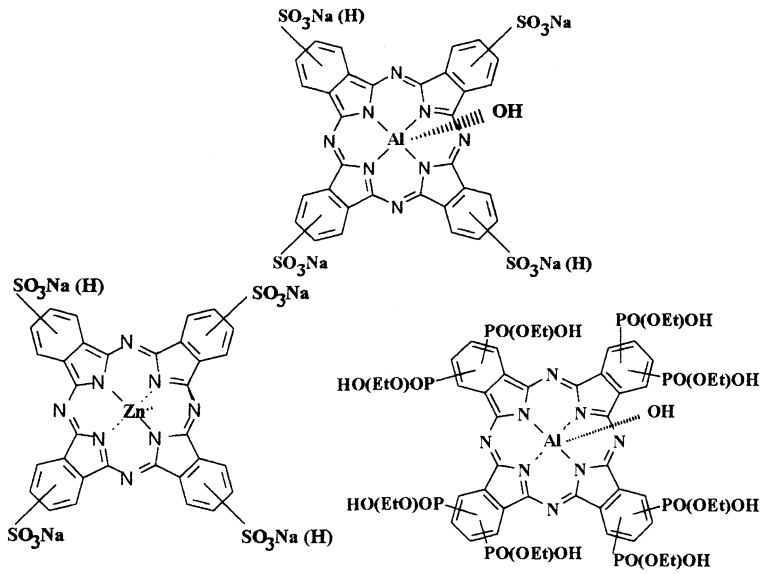

Fig. 1. Chemical formulae of tetrasulphonated Zn (top center) and Al (bottom left) phthalocyanines, and phosphonated Al phthalocyanine (bottom right).

Table 1
Composition and spectral characteristics of the studied phthalocyanines

Photosensitizer	Mean *n*	Composition (%)				λ_{max}, nm (in: DMF/HS#)	$\varepsilon_{max} \cdot 10^5$, l/mol·cm*	$k = \varepsilon_{max}/\varepsilon_{633}$ (*lg k*)**
		n=1	*n*=2	*n*=3	*n*=4			
$AlPcS_3$	3.1	—	15	50	35	677 / 676	2.00	6.5 (0.81)
$ZnPcS_2$	2.1	6	74	13	7	671 / 630-675	2.14	1.0 (0.00)
$ZnPcS_3$	3.1	—	22	51	27	675 / 660	1.69	1.3 (0.11)
$ZnPcS_4$	3.7	—	1.5	23	75.5	674 / 668	1.59	3.6 (0.56)
PAlPc	—	HOAlPc $CH_2PO(OC_2H_5)OH]_8$				695 / 691	1.27	4.5 (0.65)

DMF - dimethylformamide; HS - van Harreveld's saline; *- recorded in DMF; **- recorded in HS.

versible cessation; IEI - inhibition, activation, and new gradual inhibition until irreversible firing abolition. The dynamics of irreversible firing abolition considered as a functional sign of the cell death was of special interest. Two main types of irreversible firing cessation were observed: (a) excitatory type: firing activation followed by abrupt spike abolition, or (b) inhibitory type: gradual firing inhibition resulting in irreversible cessation of spike generation. In both cases firing did not resumed spontaneously or under additional adequate stimulation (receptor muscle extension).

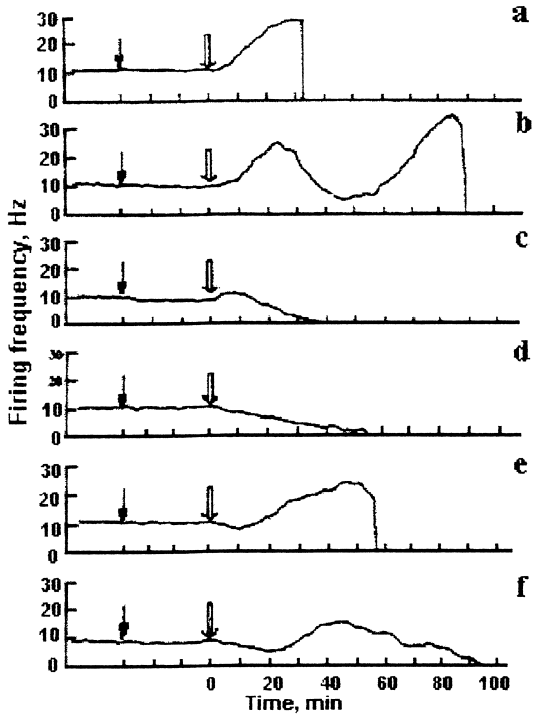

Fig. 2. Examples of the main types of neuron response dynamics to photodynamic effect : (a) E-type; (b) EIE-type; (c) EI-type; (d) I-type; (e) IE-type; (f) IEI-type. Single arrow shows the neuron staining start, double arrow - the irradiation start.

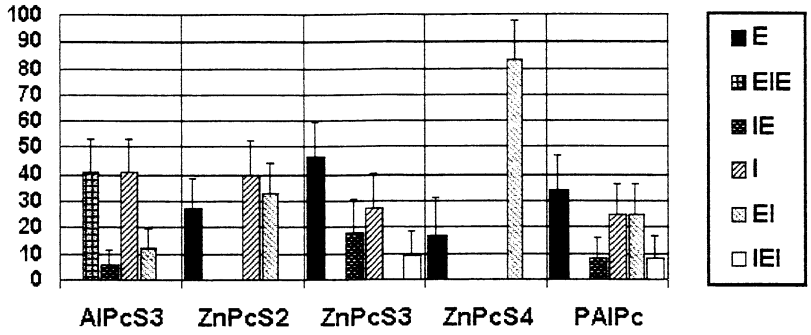

Fig. 3. The percentages of the main types of neuron responses to PD effect of different photosensitizers (standard errors are shown). E - activation followed by abrupt firing abolition; EIE - firing activation, inhibition, and new activation followed by abrupt abolition; IE - transient firing inhibition and then activation followed by abrupt abolition; I - gradual firing inhibition until irreversible cessation; EI - transient activation of firing and then gradual inhibition until irreversible cessation; IEI - inhibition, activation, and new gradual inhibition until irreversible firing abolition.

The percentage of different neuron responses depended on the type and concentration of phthalocyanines (Fig. 3). Relatively high concentrations of different PSs ($>10^{-7}$ M) predominately induced the intensive excitatory responses of E or EIE types that were observed in 72–100% of the experiments. However, the lowering of PS concentrations caused the increasing contribution of the inhibitory processes in the neuron responses which depended on the PS kind. At PS concentrations less than $5 \cdot 10^{-8} - 10^{-7}$ M different histograms of the neuron response type distributions were found to be characteristic for various PSs (Fig. 3).

As Fig. 3 shows, different patterns of neuron response distribution were observed: (i) E, IE and EIE responses causing the excitatory type of the neuron death were dominant in the cases of neuron photosensitization with $ZnPcS_3$ (62%). (ii) Inhibitory I, EI, or IEI responses with a prominent inhibition phase leading to the gradual irreversible firing abolition were observed in 73, 83, and 58% of the experiments with $ZnPcS_2$, $ZnPcS_4$, and PAlPc photosensitization, respectively. The difference or similarity in these patterns reflect presumably the different or similar mechanisms of PD effect underlying photosensitization with these PSs. For example, the response distribution patterns for neuron sensitization with $ZnPcS_2$ and PAlPc were rather similar suggesting the similar mechanism of PD effect.

Concentration dependencies

In order to compare PD efficiency of photosensitizers we studied dependencies of electrophysiologically measured neuron lifetime *T* on PS concentrations C. The comparison of functions *T(C)* for different PSs (Fig. 4, solid lines; Table 2) showed that all studied phthalocyanines were characterized with almost the same regression coefficients *b* which varied from -0.325 to -0.364. Due to the approximate equality of *b* for different phthalocyanines, i.e. due to almost the same slope of the lines in Fig. 4, the relative PD efficiency of these photosensitizers was almost entirely determined by the value *lg a*: the most effective PS had the lowest *lg a* and the appropriate line was placed in the left lower corner in Fig. 4. Accord-

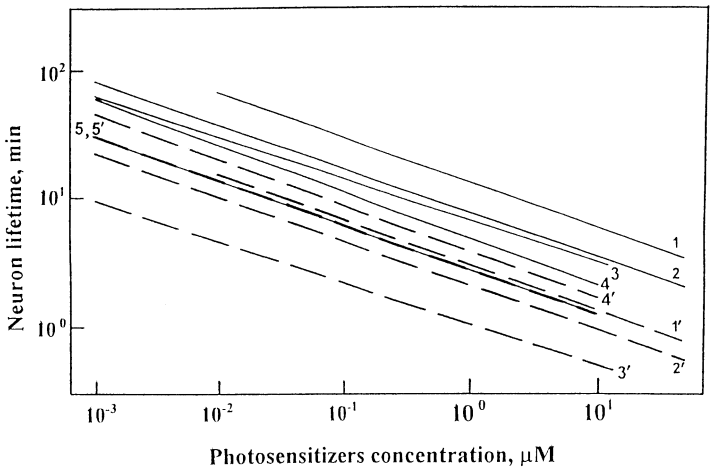

Fig. 4. Neuron lifetime *T* (min) versus concentrations *C* (μM) of different photosensitizers: (1) and (1′) - PAlPc; (2) and (2′) - $ZnPcS_4$; (3) and (3′) - $AlPcS_3$; (4) and (4′) - $ZnPcS_3$.; (5) and (5′) - $ZnPcS_2$. Solid lines - linear regression based on the experimental data for the neuron irradiation at 632.8 nm. Dashed lines are calculated with the proviso that neurons are irradiated at the absorption maximums of the appropriate photosensitizers.

ing to Fig. 4, PD efficiencies of the studied phthalocyanines decreased in the following sequence:

$$ZnPcS_2 > ZnPcS_3 > AlPcS_3 > ZnPcS_4 > PAlPc$$

$ZnPcS_2$ was found to be the most effective PS with the lowest $lg\ a = 0.44$ among the studied phthalocyanines.

The same irradiation wavelength 632.8 nm was used in all our experiments. This wavelength do not fit the absorption maximums (λ_{max}) of the studied PSs. However, it is of interest to compare PD efficiencies of photosensitizers with the proviso that cells can be irradiated at λ_{max}. Knowing the extinction ratio at these wavelengths: $k = \varepsilon_{max}/\varepsilon_{633}$ (Table 1) one can correct the graphs in Fig. 4 as they must if irradiation wavelength is λ_{max}: $lg\ a' = lg\ a - lg\ k$.

Table 2
Statistical parameters characterizing the neuron lifetime dependence on PSD concentration

Photosensitizer	Concentration range, μM	Number of experiments	Correlation coefficient, R	Regression coefficients[#]		Student's coefficient,	Fisher's coefficient,
				lg a (lg a′)	*b*	t_{st}	F
$AlPcS_3$	0.001–12	27	0.636	0.83 (0.02)	−0.325*	4.13	17.0
$ZnPcS_2$	0.001–10	24	0.579	0.44 (0.44)	−0.355	3.33	11.1
$ZnPcS_3$	0.001–10	30	0.587	0.69 (0.58)	−0.364	3.84	14.7
$ZnPcS_4$	0.001–50	16	0.771	0.89 (0.33)	−0.342	4.54	20.6
PAlPc	0.01–50	27	0.847	1.13 (0.48)	−0.350	7.96	63.4

[#]-for concentrations measured in μM.

*-this *b* value is corrected as compare to the earlier published data (9).

According to the numerical values of *lg a′* shown in brackets in Table 2, one can see that in the case of irradiation with λ_{max} PD efficiencies of the studied phthalocyanines can be ranged in the following sequence (minimal *lg a′* corresponds to minimal neuron lifetime, and, therefore, to maximal PD efficiency):

$$AlPcS_3 > ZnPcS_4 > ZnPcS_2 \approx PAlPc > ZnPcS_3$$

Therefore, Photosens have to be the most effective photosensitizer among the studied phthalocyanines in the case of cell irradiation with λ_{max}.

Discussion

Neuron lifetime, i.e. the time from the beginning of irradiation to irreversible cessation of firing, was used in the present work as a criterion of the cell death. However, cell death is a complex process involving different cellular systems: genetic apparatus, proteolytic enzymes, mitochondria, plasma membrane etc. It lasts from seconds to hours or more depending on the intensity of the applied impact [15]. It is impossible to determine precisely the cell death moment and the cell lifetime. The electrophysiological criterion of cell death, a neuron lifetime, is not a real lifetime because it does not reflect biochemical and cytological processes leading to the cell death. However, the advantage of this criterion is that it can be rather precisely measured. We assume that electrophysiological lifetime correlates with other criteria of cell death [15] and PD efficiency of PS may be characterized with the value *1/T* that is inversely proportional to *T.*

In line with the early assumption [9], PD effect of the high PS concentrations inducing firing activation followed by abrupt spike abolition, i.e. E response, was associated with PD-induced plasma membrane lesion and the following depolarization block. Actually, K.Specht and M. Rogers showed [16] that plasma membrane depolarization is an early event of $ZnPcS_2$ photosensitization in mouse myeloma cells and prolonged depolarization is known to induce the depolarization block [17]. It was also shown that in isolated crayfish neuron sensitized by 10^{-7} M Photosens the gradual firing suppression was the result of photoinjury of Ca^{2+}-storing organelles (mitochondria and/or ER) and the following Ca^{2+} release [18]. Electron microscopic study showed mitochondria damage and intracellular vacuolization in bladder carcinoma cells under PD effect of zinc phthalocyanine [19]. Therefore, firing acceleration phases in the complex neuron response dynamics could be due to the plasma membrane affection and firing inhibition phases - due to mitochondria and/or ER injury. Perhaps, in the present experiments plasma membrane lesion was responsible for the neuron responses to photosensitization with high PS concentrations and for the majority of experiments with low concentrations of $ZnPcS_3$ and $AlPcS_3$. In the case of $AlPcS_3$ photosensitization PD affection of intracellular organelles caused a transient inhibitory increment into the response dynamics producing EIE response in the major part of the experiments. Low concentrations of other PSs ($ZnPcS_2$, $ZnPcS_4$, and PAlPc), much stronger affected mitochondria and/or ER and caused firing inhibition. The highest PD efficiency of $ZnPcS_2$ could be associated with its higher lipophilicity and amphiphilicity, and hence with its ability to penetrate into different cellular membranes including ER and/or mitochondrial membranes [20,21]. However, as shown in [22,23], irradiation can cause relocalization of PS molecules in the cell. Therefore,

initial localization of PS molecules is not as important as their final sites in executing the cell killing.

The value of $b \approx 1/3$ means that one photo-excited phthalocyanine molecule induces about 3 secondary lethal lesions in the cell. These might be free radical-induced lipid peroxidation chains damaging cellular membranes. It seems that such value of *b* is a common feature of Al and Zn phthalocyanine photosensitization. Moreover, it is possible that different PS classes may be characterized with different *b.* For example, it was recently shown, that *b* varied from 0.22 to 0.30 in the case of neuron photosensitization with 6 different deuteroporphyrin derivatives (Uzdensky et al., submitted for publication).

In conclusion, we have demonstrated that sulphonated Al and Zn phthalocyanines are very potent photosensitizers, killing nerve cell at nanomolecular concentrations. $ZnPcS_2$ was shown to be the most effective upon irradiation with He-Ne laser (633 nm). However, the calculation of relative PD efficiencies with the proviso that cell are irradiated with the absorption maximum wavelength showed $AlPcS_3$ to be the most effective PS. All Al and Zn phthalocyanines seems to affect biological membranes in a similar manner creating about 3 secondary lesions upon one photon absorption.

Acknowledgments

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