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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Photosensibilization with Endogenous Riboflavin of the Isolated Mechanoreceptor Neuron and Satellite Glial Cells of the Crayfish *Astacus leptodactilus*

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Abstract—The photodynamic effect of the exogenous riboflavin on the mechanoreceptor neuron and satellite glial cells was studied in the isolated stretch receptor of the river crayfish. It was shown that the photodynamic action of exogenous riboflavin produced lesion of the cytoplasmic membrane and irreversible cessation of the neuron impulse activity, disturbance of integrity of the plasma membrane of glial cells, and development of necrotic processes in them. It also induces apoptosis of the glial cells. A disturbance of bioenergetic processes in the neuron and development of apoptosis in the glial cells was observed with a 4-h delay after the photoinduced cessation of the neuron impulsation. Riboflavin is known to be a photosensitizer of the first kind, which generates superoxide-anion during illumination. Its photodynamic effect on the neuron was essentially lower than the photodynamic effect of the earlier studied photosensitizers of the second kind—porphyrines, chlorines, and phthalocyanines. They produced the cell lesions that did not developed cessation of impulsation, as this took place in the case of photosensitizers of the first kind.

INTRODUCTION

Until the last third of the last century, effect of the visual light on animal cells was studied to the lesser degree than a more expressive effect of the ultraviolet irradiation [1–3]. However, for the last decades, especially after the appearance o lasers, much information has been obtained that the ecologically significant visual light is able to change the physiological state and even to damage the animal non-pigmented cells that have no special photoreceptor apparatus [4–6]. A great attention was drawn to the red light diapason that did not damage the cells, but stimulated and supported their vitality [6].

It was shown in works [7, 8] that the blue laser irradiation changed impulse activity and inactivated irreversibly nerve cells at a long-term action. The spectrum of this action had "flavin maximum" at 460 nm [9], and ultrastructural studies showed [10] that the greatest damage involved inner mitochondria membranes able to absorb the visual light, inserted in which are oxidative-reductive enzymes carrying chromophore coenzyme groups—flavins, hems, iron-sulphur centers. Since this is accompanied by inactivation of flavoprotein succinate dehydrogenase [7] playing a most important role in the cell bioenergetics, it was suggested that the flavin compounds are endogenous photosensitizers in the non-pigmented neurons [7–11]. Therefore, it seemed interesting to elucidate reactions of nerve cells to photosensitization by exogenous flavins.

Flavins, particularly riboflavin (vitamin B_2), can sensitize photoinjury of various proteins and membranes in cells [12]. Riboflavin is known to be photosensitizer (PS) of the first kind, which generates superoxide-anion [13] at illumination. This distin-

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guishes it from porphyrin PS of the second kind used in the photodynamic therapy, the mechanism of their phototoxicity being due to photogeneration of the singlet oxygen [14, 15]. Earlier we studied reactions of isolated crayfish mechanoreceptor neurons to the photodynamic action (PDA) of various hematoporphyrins, deiteroporphyrins, phthalocyanines, chlorines, and other dyes [16–20]. Therefore, the second goal of this work was comparison of reactions of these cells to the riboflavin PDA with the earlier studied PDA of the photosensitizer of the second kind.

Mechanoreceptor neurons, like other nerve cells, are surrounded by the glial sheath playing an important role in providing the neuron life activity and function and in maintaining their survival at damaging actions [21]. Reactions of the glial cells to PDA practically are not studied. Therefore, the third goal of this work was the study of the riboflavin PDA on the glial cells surrounding the mechanoreceptor neuron.

MATERIALS AND METHODS

The object of the study was an isolated slowly adapting mechanoreceptor neuron (MRN) of the river crayfish *Astacus leptodactilus*. The stretch receptor of the crayfish was prepared by the method of Wiersma *et al.* [22] and transferred into a bath with van Harreveld solution containing (mM): 205 NaCl, 5.4 KCl, 0.24 $NaHCO_3$, 5.4 $MgCl_2$, 13.5 $CaCl_2$, pH 7.2–7.4. Action potentials (AP) were recorded extracellulary from the axon with the glass sucking electrode and were amplified by an UU-90 biopotential amplifier (Institute of Experimental Medicine, Leningrad). The impulse frequency was recorded using an MFU-1 frequency meter (Institute of Experimental Medicine, Leningrad) and an N-39 self-recorder (ZIP, Krasnodar). The receptor muscles were stretched in such a way that MRN generated impulses with frequency at the order of 10–12 Hz. After preparation, the control recording of the AP frequency was performed for 30 min, then the preparation was incubated for 30 min with 10^{-5} or 10^{-4} mole/l riboflavin (Reanal, Hungary) and irradiated with the blue light of an incandescence lamp with a SZS-9 light filter (400–550 nm, maximum at about 480–500 nm). The irradiation intensity measured with an IMO-2N apparatus (Etalon, Volgograd) amounted to 0.25 Wt/cm^2.

The frequency dynamics of the neuron AP was recorded continuously until the irreversible cessation of impulsation for detection of all phases of its reaction to PDA. The irreversible impulse cessation was considered as a functional index of the neuron death. The neuron lifespan T was measured in frequencygram from beginning of the irradiation till the moment of the impulse cessation.

To determine activity of succinate dehydrogenase (SDH), the van Harreveld solution in the bath was replaced by the incubation mixture of the follows composition: 1 mg/ml Tetrazolium Nitroblue (Chemapol, Czechoslovakia), 0.05 M sodium succinate (Reakhim, USSR), 5 mM $MgCl_2$, 10 mM NaN_3 in 0.05 M Tris-HCl buffer, pH 7.2 [11, 23]. The incubation mixture was prepared at once before the experiment. The optimal incubation time was 50 min at 23–24°C. After the incubation the preparation was washed with saline, fixed for 20 min with 0.1% glutaraldehyde and embedded in glycerol. The functioning neurons not submitted to PDA served control. The preparations were photometried using an FMEL-1 photometric apparatus (LOMO, Leningrad) installed in an Amplival microscope (Carl Zeiss, Jena, Germany).

The fluorescence microscopy study was performed using a LUMAM-IZ microscope (LOMO, Leningrad). The preparations were photographed to a Kodak ProFoto II 400 film or used a Nikon Coolpix 955 digital camera (Nicon Corporation, Japan).

To study intactness of plasmalemma after the neuron photoinactivation and 3-fold washing with van Harreveld solution, 20 μmole/l propidium iodide (Sigma, USA) that penetrated only into the necrotic cells with the injured plasma membrane was added into the bath. The symmetric neurons generating AP with the initial frequency for the same time as experimental neurons served control. The neurons were fixed with 0.1% glutaraldehyde immediately after the photoinduced cessation of the impulse activity or after 4 h. Then, after the 3-fold washing the cells were incubated for 15 min with the Hoechst 33342 dye (20 μM) that selectively fluorochromies nuclear chromatin and embedded in glycerol. The area of the neuron nucleus was calculated by the ellipse formula with axes a and b: $S = \pi ab/4$.

The results of experiment were treated statistically using Student criterion by the standard methods [24]. The data are presented as the mean value ± standard error ($\bar{x} \pm S_{\bar{x}}$).

RESULTS

The neuron reactions to riboflavin photosensitization. The exogenous riboflavin (10^{-4} M) itself increases inactivation of isolated neurons. Whereas control unstained neurons after their isolation generated impulses in 10–15 h, in the presence of riboflavin they functioned steadily in the darkness only for 4–5 h, then their activity was gradually inhibited and stopped (Fig. 1a; Table 1). On illumination of MRN with a bright blue light in the absence of riboflavin the MRN impulse activity ceased, on average, in 2.7 h (Fig. 1b; Table 1). The phototoxicity of the blue light might be due to photosensibilization of the neuron with endogenous flavins [8, 11].

In experiments with photodynamic riboflavin action a prominent MRN inactivation was observed only at a concentration exceeding 10^{-5} M. At such concentration the MRN lifespan amounted, on average, to 122 min, while at 10^{-4} M, 37 ± 3 min (Table 1). The neuron inactivation occurred as a result of a gradual decrease of the impulse frequency until to the complete cessation of the impulse activity. At the 10^{-4} M riboflavin PDA the MRN impulsation was first accelerated temporarily, then was inhibited (Figs. 1c, 1d).

Both at the moment of the end of the neuronal activity due to photosensibilization with 10^{-4} M riboflavin and after 4 h the cytoplasmic membrane was damaged in about half of the neurons, which allowed propidium iodide to penetrate into the cell and to fluorochrome the nucleus (Fig. 2; Table 2). However, at this moment, the SDH activity in the MRN body did not differ statistically significantly from control, i.e., the bioenergetic processes in mitochondria were not essentially disturbed. The SDH activity decreased statistically significantly twice only after 4 h (Fig. 3; Table 2), which indicates a decrease of the ATP synthesis in the cell. For the first 4 h after the riboflavin PDA no essential changes of the size and morphology of the neuron nuclei, which are characteristic in particular of apoptosis (pyknosis and fragmentation of nuclei), were observed (Fig. 2; Table 2).

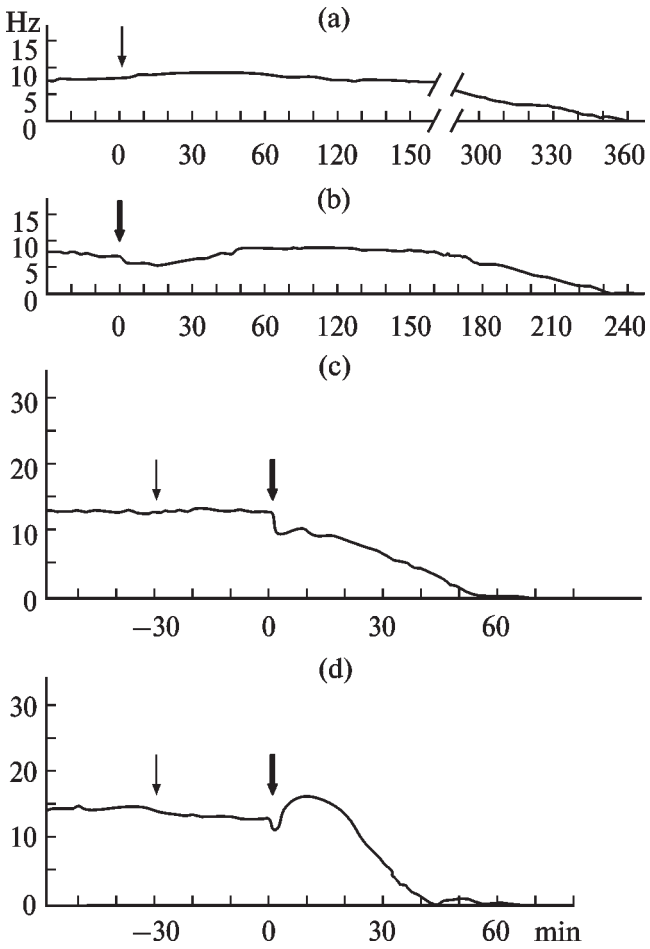

Fig. 1. PDA of riboflavin on the neuron impulse activity. (a) Dynamics of the neuron impulse response to the dark action of 100 μM riboflavin, (b) dynamic of the neuron impulse response to illumination with blue light (400–550 nm, 0.25 Wt/cm^2); (c), (d) two examples of dynamics of the neuron impulse response to a combined action of 100 μM riboflavin and the blue light. *Abscissa*: time (min), *ordinate*: AP frequency (Hz). *Thin arrow* demonstrates the moment of the riboflavin addition, *bold arrow*—start of irradiation.

The satellite glia reactions to riboflavin photosensitization. Damage of the plasma membrane and changes of the nucleus morphology of the satellite glial cells surrounding MRN can be observed in the same preparations of the crayfish stretching receptor. The photosensitization with 10^{-4} M riboflavin led to the cytoplasmic membrane lesion and necrosis in about a half of the glial cells around the cell body and in 40% of cells surrounding axon. The

Table 1. Photodynamic inactivation of neuron with riboflavin

Conditions of experiment	The number of experiments	Lifespan (min)
1. Blue light, 0.25 Wt/cm^2	6	162 ± 28
2. Riboflavin, 10^{-4} M	5	288 ± 36
3. Riboflavin, 10^{-5} M + blue light	5	122 ± 21
4. Riboflavin, 10^{-4} M + blue light	23	37 ± 3**

Note: Asterisks mark statistically significant differences from the variants 1, 2, and 3, $p < 0.01$.

lesion was observed at once after the photoinduced cessation of the MRN impulsation (Figs. 2 and 4; Table 2); the percent of the damaged cells did not change after 4 h. Unlike necrosis, the number of apoptotic glial cells with fragmented nuclei increased 3 times statistically significantly 4 h after the MRN inactivation and PDA cessation (Fig. 4; Table 2).

DISCUSSION

Our data have shown that the exogenous riboflavin can sensitize photodamage of neurons and satellite glial cells. The death of the photosensitized cells might result from photoinjury of various cell membranes and proteins [12]. The oxidized flavins are known to absorb well the blue light with maximum at around 450 nm. Interaction of the photoexcited flavin molecules of with oxygen in the presence of electron donors, for example cytochromes, produces formation of water. The intermediate products are the superoxide-anion and hydrogen peroxide [13]. During protein photosensitization with riboflavin, amino acids, first of all tryptophan, are photooxidated [25]. It was suggested [26] that various active oxygen forms participated in these reactions, such as $O_2^{\bullet}$, $^1O_2$, H_2O_2, and $OH^{\bullet}$, but the quantum yield of the superoxide-anion turned out to by much higher [27]. Hence, flavins are photosensitizers of the first kind unlike photosensitizers of the second kind used in the photodynamic therapy, porphyrines, phthalocyanines or chlorines, generating cytotoxic singlet oxygen at illumination [12]. The flavin substances are localized mainly in the mitochondrial inner membrane and matrix. But they are also detected in the endoplasmic reticulum, nuclear envelope, and cytoplasmic membrane [28, 29]. Therefore, on irradiation with a sufficiently intensive blue light the endogenous flavins can sensitize the photoinjury of various cell membranes [8, 10].

The presented experiments have shown that exogenous flavins also can sensitize photoinjury of cells, particularly of neurons and satellite glia. This situation in principle appear in the organism at overdose of vitamin B_2 and the a long stay at a bright light. In this case, peripheral nerve elements will be vulnerable.

The photosensitizing ability of riboflavin, photosensitizer of the first kind, was essentially lower than the previously studied photosensitizers of the second kind, which photogenerate singlet oxy-

Table 2. Photodynamic action of riboflavin (10^{-4} M) on metabolic and morphological characterizations of the mechanoreceptor neuron and the surrounding glia

Time after photosensitization, (h)	The number of neurons with the damaged membrane (%)	Area of the neuron nucleus (μm^2)	SDH activity (relat. units)	Percent of glial cell with the damaged membrane		Percent of glial cells with fragmented nuclei
				around axon	around nucleus	
Control	10 ± 7 (19)	720 ± 70 (16)	0.34 ± 0.04 (16)	22 ± 4 (26)	16 ± 3 (25)	3.2 ± 0.7 (16)
0	55 ± 11** (22)	620 ± 64 (12)	0.36 ± 0.04 (12)	47 ± 6** (22)	43 ± 6** (22)	5.8 ± 1.4 (35)
4	30 ± 19* (8)	700 ± 90 (5)	0.18 ± 0.01** (7)	48 ± 9** (9)	39 ± 9* (9)	16.7 ± 6.0* (11)

Note: Asterisks mark statistically significant differences from control: one—$p < 0.05$, two—$p < 0.01$. In parentheses—the number of experiments.

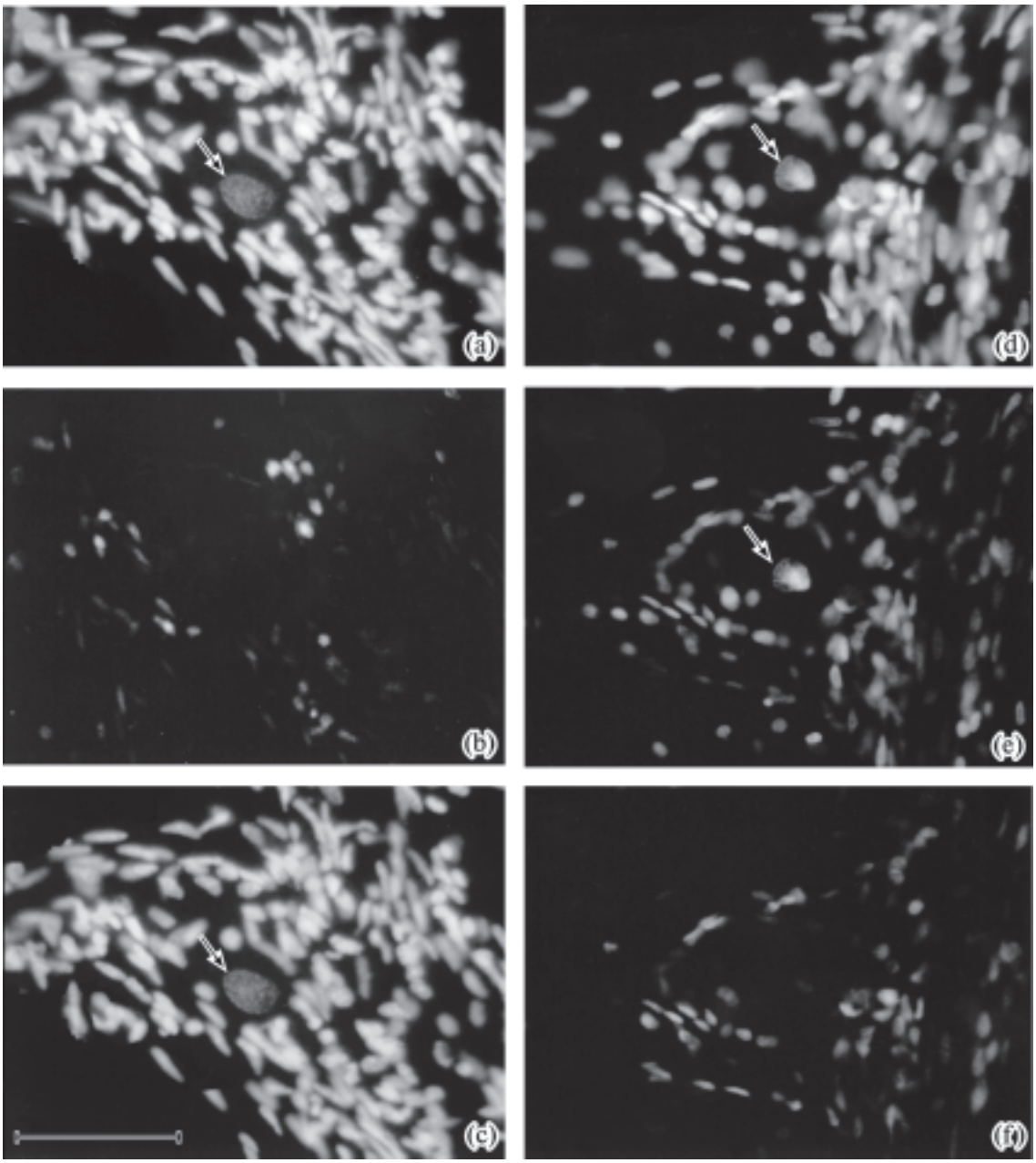

Fig. 2. Nuclei of the neuron and of its surrounding glial cells after the 10^{-4} M riboflavin PDA. Double staying with fluorochromes Hoechst 33242 (blue fluorescence) and propidium-iodide (red fluorescence). (a)–(c) Control preparation that is not submitted to photodynamic action; (d)–(f) photosensitized preparation; (a), (d) the common fluorescence of injured and non-injured nuclei stained with both fluorochromes. The computer (red-green-blue) RGB-splitting of the image was used for visualization of nuclei of different colors. Only red nuclei of the necrotic cells stained with propidium-iodide are presented in the "red channel"(b, e), while in the "blue channel"(c, f)—the rest of the nuclei. *Arrow* indicates the neuron nucleus. Scale bar—50 μm. Ob. ×40.

gen,—phthalocyanines [17], porphyrines [18] or chlorines [20]. The neuron photoinactivation with riboflavin required its micromolar concentrations (10^{-5}–10^{-4} M), whereas the above photosensitizers were effective at concentrations 3–5 orders lower. The blue light intensity in these experiments (0.25 Wt/cm ^{2}) was nearly the same as that of the red light the works referred to above (0.3 Wt/cm^2),

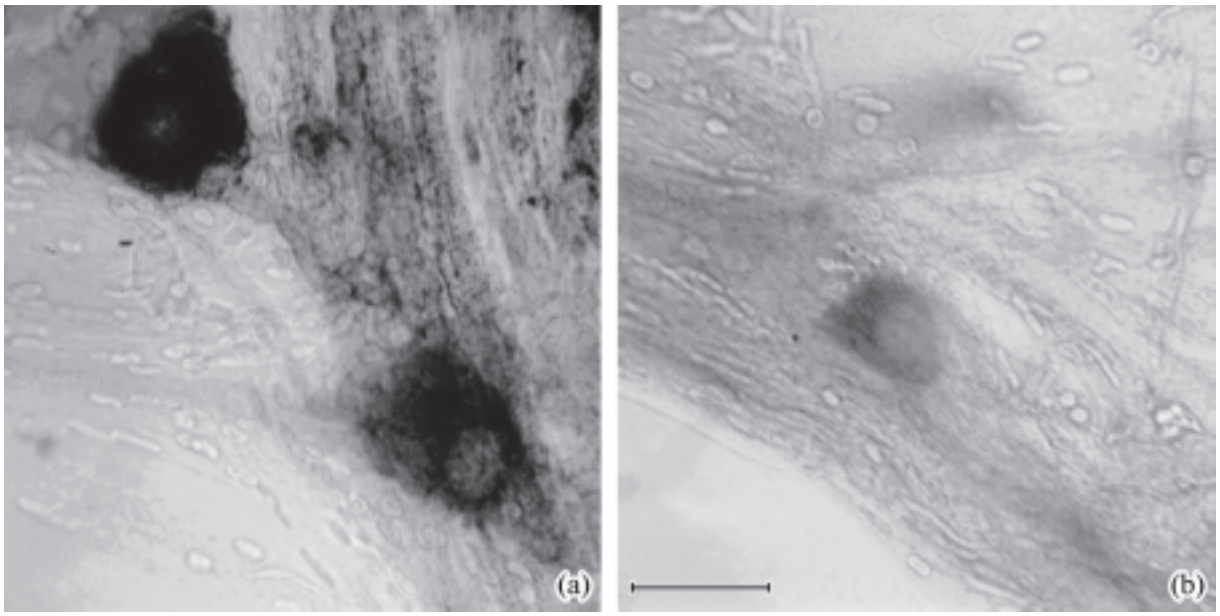

Fig. 3. The photodynamic inactivation of succinate dehydrogenase in the crayfish stretch receptor. (a) control preparation, (b) preparation photosensitized by 10^{-4} M riboflavin. Intensity of staining is significantly reduced 4 h after the photoinduced cessation of impulse activity. Bodies of slowly and quickly adapting neurons are stained with formazan. Scale bar—50 μm. Ob. ×40.

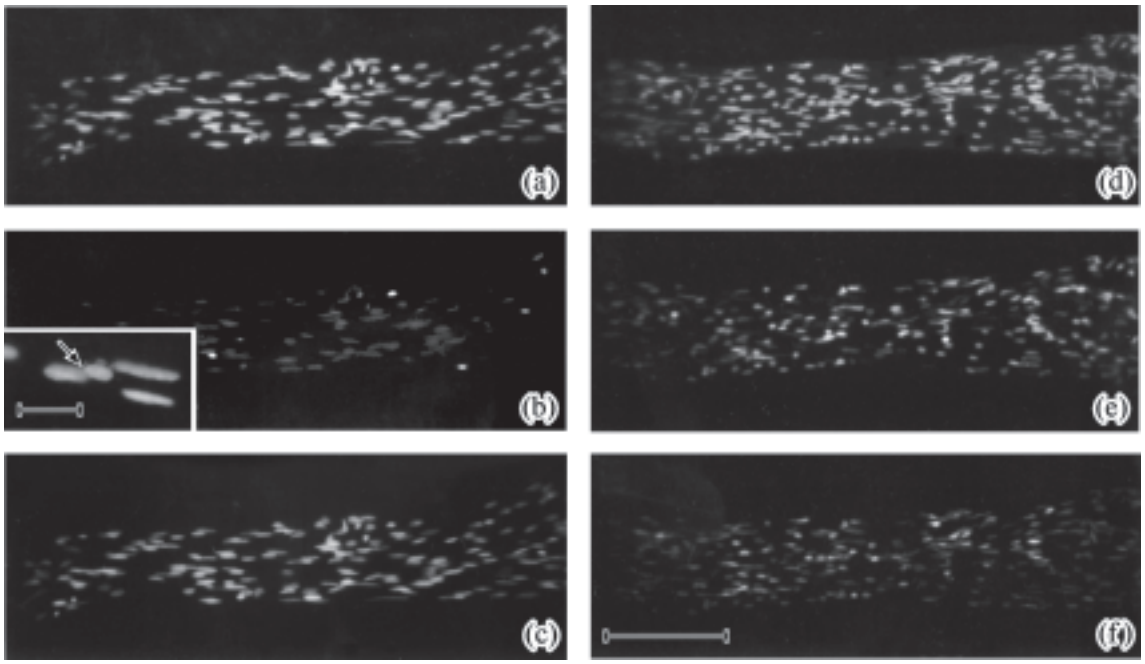

Fig. 4. The photodynamic action of 10^{-4} M riboflavin on glial cells surrounding axon. Double staying with fluorochromes Hoechst 33242 (blue fluorescence) and propidium-iodide (red fluorescence). (a)–(c) Control preparation that is not submitted to photodynamic action; (d)–(f) photosensitized preparation; (a), (d) the common fluorescence of injured and non-injured nuclei stained with both fluorochromes. The computer (red-green-blue) RGB-splitting of the image was used for visualization of nuclei of different colors. Only red nuclei of the necrotic cells stained with propidium-iodide are presented in the "red channel"(b, e), while in the "blue channel"(c, f)—the rest of the nuclei. Scale bar—50 μm. In the insert, *arrow* indicates fragmented nucleus of apoptotic glial cell. Scale bar—10 μm. Ob. ×40.

while the riboflavin molar extinction in van Harreveld solution (pH 7.4) with the maximum at 443 nm (125 000 l mole^{-1}/cm^{-1}) was of the same order or only one order lower than the extinction of the above-mentioned PS (Bragin and Usdensky, unpublished data). Therefore, the lower riboflavin effect can be suggested to be due not to absorption of the lower number of photons, but to the lower cytotoxity of the superoxide-anion as compared with singlet oxygen.

The inactivation of riboflavin-photosensitized neurons occurred by the way of a gradually inhibition of the impulse activity up to its complete cessation (Fig. 1), as this was observed by at low concentrations of other PS [17–20, 30]. This process was coupled with photoinjury of intracellular Ca^{2+}-storing organelles (mitochondria and endoplasmic reticulum), release of Ca^{2+} from them, and subsequent neuronal hyperpolarization due to activation of Ca^{2+}-depended K^+-channels [19, 30]. The appearance of an additional phase of acceleration of impulsation in the MRN reaction to the PDA of 10^{-4} M riboflavin (Figs. 1c, 1d) can be due to the direct photoaction on the plasma membrane and its depolarization [7, 8, 16, 30]. The damage of the plasmalemma integrity is also indicated by propidium-iodide penetration into the neuronal nuclei, which suggests development of necrotic processes in MRN. After cessation of irradiation the MRN portion with the damaged plasma membrane did not change. Hence, the plasmalemma damage at the riboflavin photosensitization occurred only during irradiation, rather than after it, as in the case of the phthalocyanine photodynamic action [19], which seemed to initiate a chain of secondary darkness reactions continuing after the cessation of irradiation. This appears to be another cause of the higher PDA of the photosensitizers of the second kind as compared with riboflavin.

Interestingly, the bioenergetic processes were disturbed not in the course of photosensitization with riboflavin, but as late as 4 h after it. This is not consistent with the hypothesis that Ca^{2+} output from photodamaged mitochondria can induce inhibition and cessation of the MRN impulse activity. The exogenous riboflavin is possible to penetrate better into endoplasmic reticulum membranes than into mitochondria. At the lesion of the reticulum cisterns, Ca^{2+} can be released into the cytosol and activate Ca^{2+}-dependent K^+-channels, which inhibits impulsation [31].

The percent of necrotic glial cells with the damaged membrane approximately corresponded to the proportion of necrotic neurons (Table 2). We did not observe pyknosis and fragmentation of neuronal nuclei characteristic of apoptosis [32]. But under the effect of the riboflavin PDA, apoptosis of glial cells, unlike neurons; it developed 4 h, and even more hours, after the MRN inactivation and the end of photosensitization. No apoptosis of the mechanoreceptor neurons was also revealed at the PDA of other photosensitizers [21].

Thus, the riboflavin photodynamic action causes the MRN functional inactivation, lesion of the cytoplasmic membrane of neurons and glial cells and formation in them of necrotic processes. It also induces apoptosis of glial cells. The disturbance of bioenergy processes in MRN and development of apoptosis occurs not earlier than 4 h after the photoinduced cessation of impulsation of the neurons.

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