Reactive oxygen species produced by photodynamic effect induced calcium signal in neurons and astrocytes

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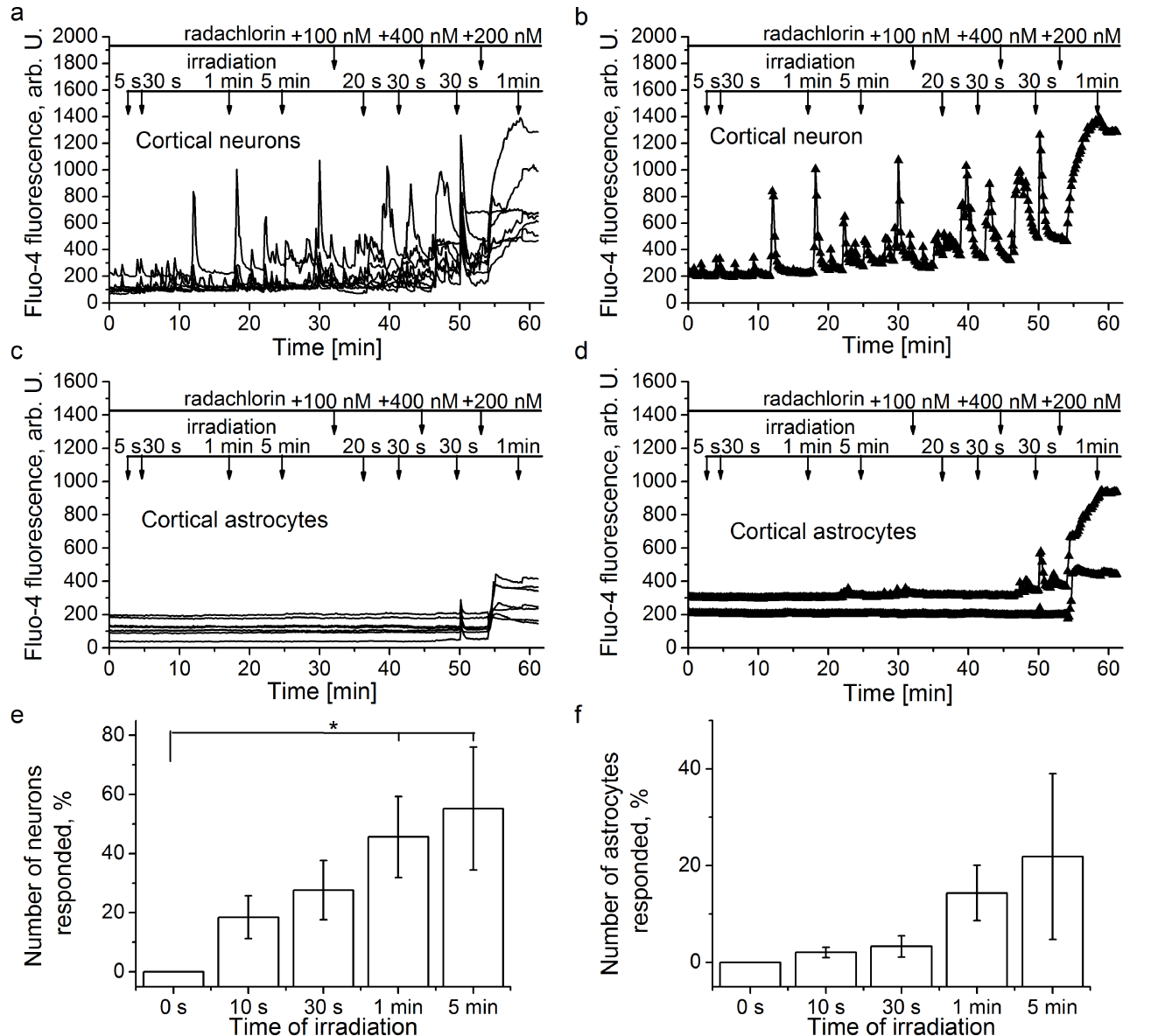
Abstract

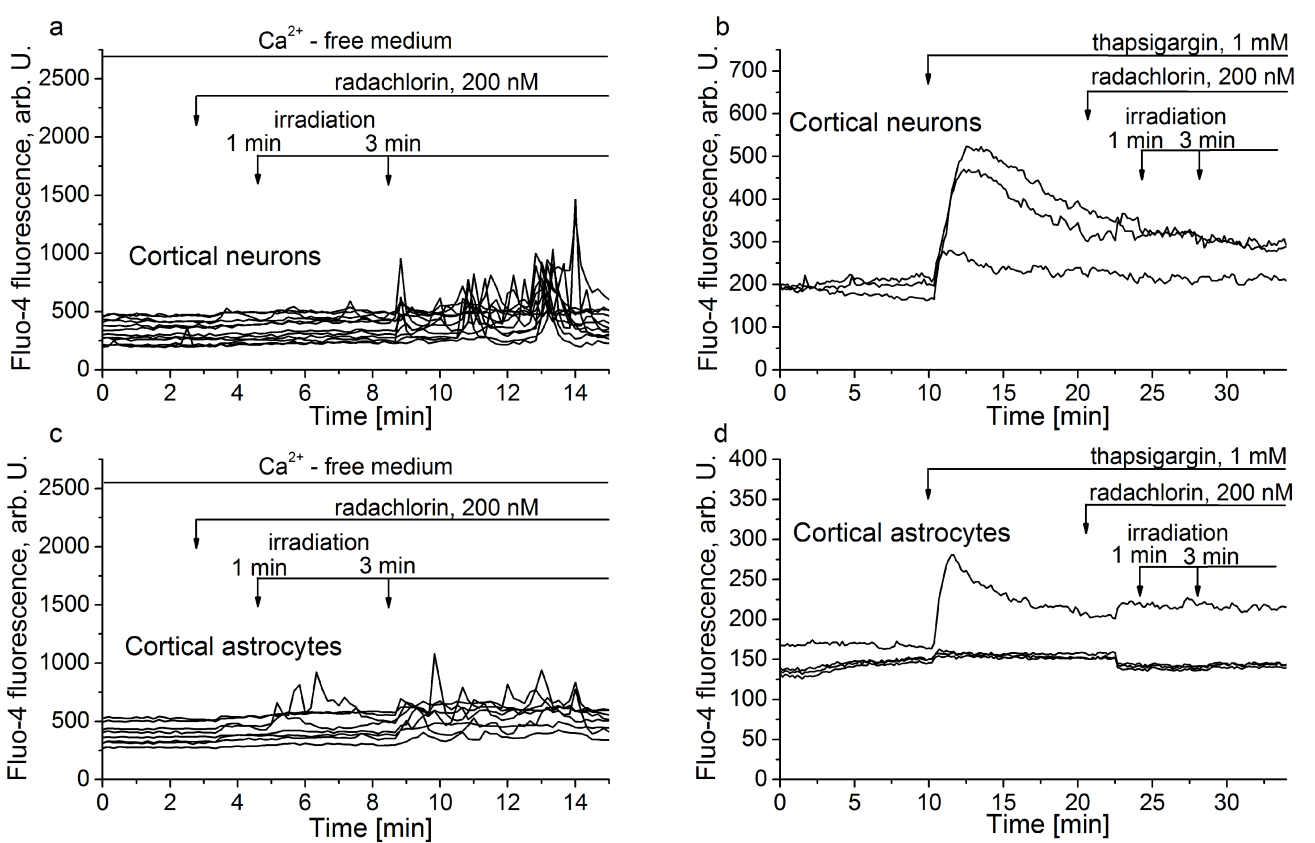
Photodynamic therapy (PDT) leads to production of reactive oxygen species (ROS) and cell destruction due to oxidative stress. We used photodynamic effect of photosensitizer radachlorin to unravel the effect of photo-induced oxidative stress on the calcium signal and lipid peroxidation in primary culture of cortical neurons and astrocytes using live cell imaging. We have found that irradiation in presence of 200 nM of radachlorin induces calcium signal in primary neurons and astrocytes. Photo-induced neuronal calcium signal depends on internal calcium stores as it was still observed in calcium free medium and could be blocked by depletion of endoplasmic reticulum (ER) stores with inhibitor of sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) thapsigargin. Both inhibitor of phospholipase C activity U73122 and water soluble analogue of vitamin E Trolox suppressed calcium response activated by PDT. We have also observed that the photodynamic effect of radachlorin induces lipid peroxidation in neurons and astrocytes. This data demonstrate that lipid peroxidation induced by PDT in neurons and astrocytes leads to activation of phospholipase C that results in production of inositol 1,4,5-trisphosphate (IP3).

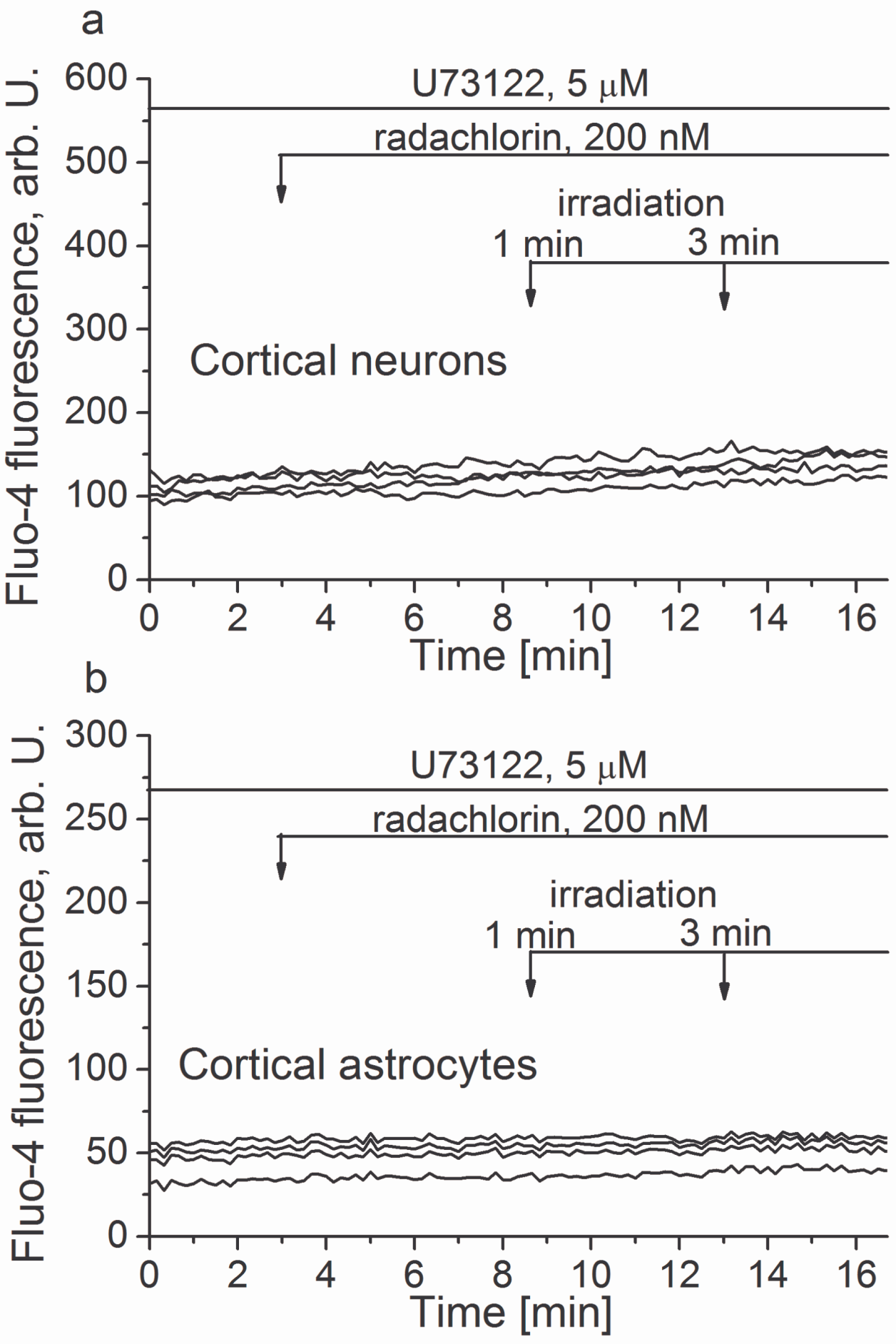
Keywords: photodynamic effect, neurons, astrocytes, calcium signaling, radachlorin

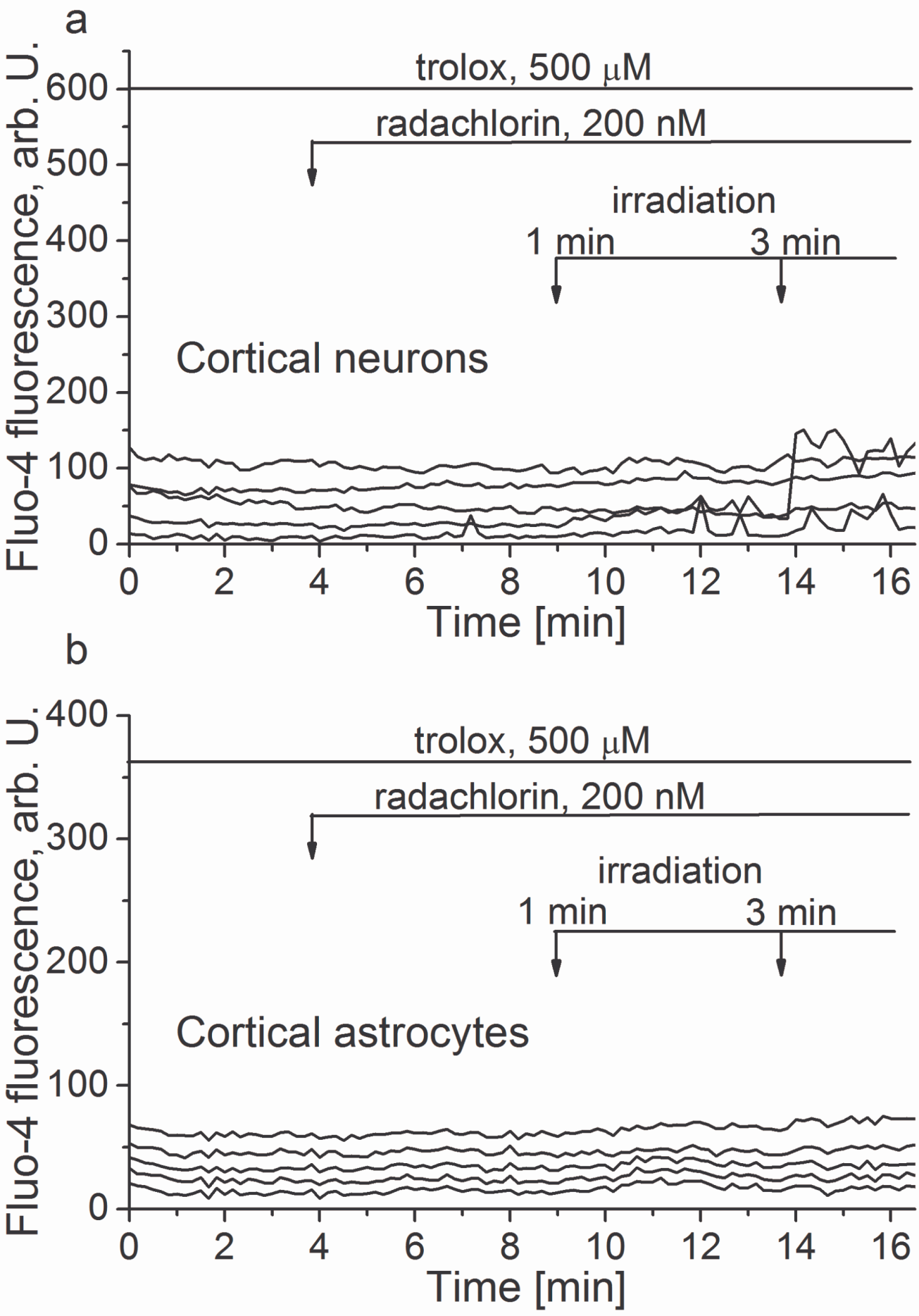
Acknowledgements:

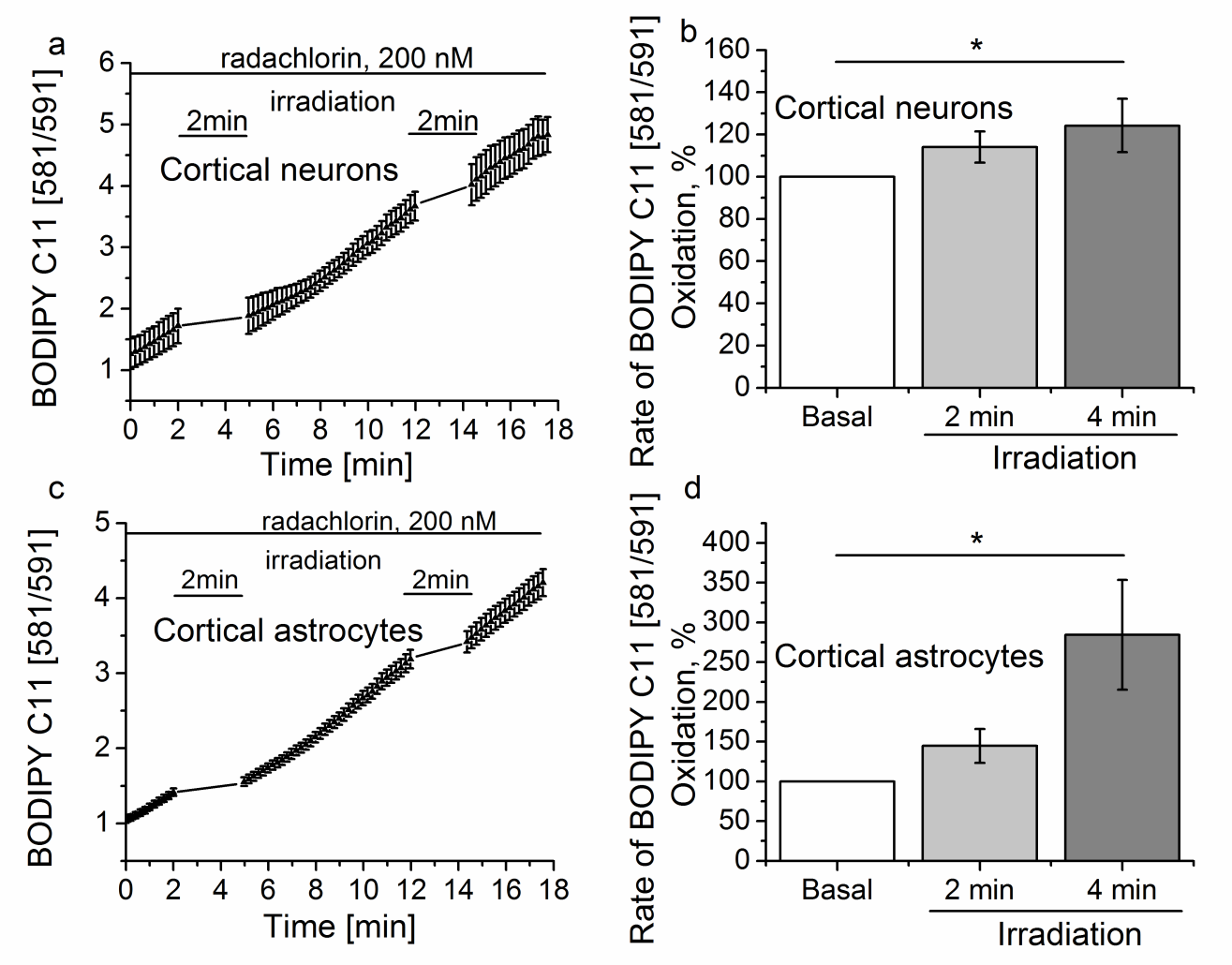
The work was supported by the Russian Science Foundation (project No. 14-15-00068) and Russian Foundation for Basic Research (project No. 17-04-01728). A.B. Uzdensky was supported by the Ministry of Education and Science of Russian Federation (project No. 6.4951.2017/VU).


a
Fluo-4 fluorescence, arb. U.
radachlorin +100 nM +400 nM +200 nM
irradiation
5 s 30 s 1 min 5 min 20 s 30 s 30 s 1min
Cortical neurons
Time [min]
b
Fluo-4 fluorescence, arb. U.
radachlorin +100 nM +400 nM +200 nM
irradiation
5 s 30 s 1 min 5 min 20 s 30 s 30 s 1min
Cortical neuron
Time [min]
c
Fluo-4 fluorescence, arb. U.
radachlorin +100 nM +400 nM +200 nM
irradiation
5 s 30 s 1 min 5 min 20 s 30 s 30 s 1min
Cortical astrocytes
Time [min]
d
Fluo-4 fluorescence, arb. U.
radachlorin +100 nM +400 nM +200 nM
irradiation
5 s 30 s 1 min 5 min 20 s 30 s 30 s 1min
Cortical astrocytes
Time [min]
e
Number of neurons responded, %
*
0 s
10 s
30 s
1 min
5 min
Time of irradiation
f
Number of astrocytes responded, %
0 s
10 s
30 s
1 min
5 min
Time of irradiation


a
Ca2+ - free medium
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical neurons
Fluo-4 fluorescence, arb. U.
Time [min]
b
thapsigargin, 1 mM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical neurons
Fluo-4 fluorescence, arb. U.
Time [min]
c
Ca2+ - free medium
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical astrocytes
Fluo-4 fluorescence, arb. U.
Time [min]
d
thapsigargin, 1 mM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical astrocytes
Fluo-4 fluorescence, arb. U.
Time [min]


a
Fluo-4 fluorescence, arb. U.
600
500
400
300
200
100
0
U73122, 5 μM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical neurons
0
2
4
6
8
10
12
14
16
Time [min]
b
Fluo-4 fluorescence, arb. U.
300
250
200
150
100
50
0
U73122, 5 μM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical astrocytes
0
2
4
6
8
10
12
14
16
Time [min]


a
trolox, 500 μM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical neurons
Fluo-4 fluorescence, arb. U.
Time [min]
b
trolox, 500 μM
radachlorin, 200 nM
irradiation
1 min
3 min
Cortical astrocytes
Fluo-4 fluorescence, arb. U.
Time [min]


a
radachlorin, 200 nM
irradiation
2min
2min
Cortical neurons
BODIPY C11 [581/591]
Time [min]
b
Cortical neurons
Rate of BODIPY C11 [581/591] Oxidation, %
Basal
2 min
4 min
Irradiation
*
c
radachlorin, 200 nM
irradiation
2min
2min
Cortical astrocytes
BODIPY C11 [581/591]
Time [min]
d
Cortical astrocytes
Rate of BODIPY C11 [581/591] Oxidation, %
Basal
2 min
4 min
Irradiation
*

Introduction

Photodynamic therapy (PDT) is a method used for destruction of brain tumors. PDT is based on production of reactive oxygen species resulting in oxidative stress and death of the cells stained by photosensitizer [1]. Radachlorin is a chlorine derivative photosensitizer currently used for treatment of various types of cancer. It was shown to have a lot of advantages such as rapid accumulation in tumor tissue, high affinity to tumor cells, and fast elimination from the body [2]. However, the mechanism of responses of healthy cell to photo-damage induced by radachlorin still remains unclear.

One of the major effects of photodynamic therapy is induction of the reactive oxygen species production. Excessive production of ROS can lead to oxidative stress followed by triggering of the cell death [3]. However, enzymatic and non-enzymatic free radicals production can play physiological role [4] and can be a stimulus for calcium signaling [5, 6].

Calcium ion play important role in regulation of the intracellular processes and cell to cell communication [7]. This intracellular messenger is specifically important in excitable cells, including neurons and astrocytes. Calcium signaling in the brain cells controls most of the physiological processes and also regulates mechanism of the cell death [7].

The ability of PDT to induce an increase in intracellular calcium was shown previously [8–10]. PDT could lead to increase in $[Ca^{2+}]_c$ through activation of calcium channels in plasma membrane or through release of Ca^{2+} from internal store of ER via activation of IP3 receptors or PDT mediated SERCA inhibition. The mechanism of the photo-induced calcium signal depends not only on photosensitizer used and its intracellular localization but on the type of the cells treated [11, 12]. The mechanism of photodynamic effect and specifically the photodynamic effect of radachlorin on calcium signal of neurons and glial cells is poorly studied.

In this work, we investigated the mechanism of calcium responses in primary co-culture of neurons and astrocytes in response to photodynamic treatment with radachlorin.

Materials and methods

Primary co-culture preparation

Mixed cultures of hippocampal, cortical neurons and glial cells were prepared as described previously [13, 14] from Sprague-Dawley rat pups 2–4 days postpartum (UCL breeding colony). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air for a minimum of 12 days before experimental use to ensure the expression of receptors. Neurons were easily distinguishable from glia: they appeared phase bright, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer. Cells were used at 12–18 days in vitro unless differently stated. All animal experiments were performed according to approved guidelines.

Imaging $[Ca^{2+}]_c$ and Lipid Peroxidation

For measurement of $[Ca^{2+}]_c$, primary co-cultures of neurons and astrocytes were loaded for 40 min at room temperature with 5 μM Fluo-4 AM with 0.005% pluronic in a HBSS (containing 156 mM NaCl, 3mM KCl, 2mM MgSO4, 1.25mM KH2PO4, 2 mM CaCl2, 10 mM glucose and 10 mM HEPES, pH adjusted to 7.35 with NaOH. Ca^{2+}-free HBSS also contained 0.5 mM EGTA.

Fluorescence measurements were obtained on an epifluorescence inverted microscope equipped with a 20x fluorite objective. $[Ca^{2+}]_c$ was monitored in single cells using excitation light provided by a xenon arc lamp, the beam passing monochromator at 490 nm (Cairn Research, Kent, UK). Emitted fluorescence light was reflected through a 515-nm longpass filter to a cooled CCD camera (Retiga; QImaging) and digitized to 12-bit resolution.

For measurement of the lipid peroxidation rate, astrocytes were loaded with 5 μM BODIPY C11 [581/591] for 30 min. BODIPY C11 fluorescence was measured using a Zeiss 710 CLSM confocal microscope. BODIPY C11 [581/591] was excited using the 488 and 563 nm laser line, and fluorescence measured from 505 to 550 nm and 570 and 630 nm.

All imaging data were collected and analyzed using software Image J.

Photodynamic treatment

After control registration of baseline of Fluo-4 fluorescence radachlorin (200 nM) was added to the cells and after incubation (5 min) the cells were irradiated with laser beam (654 nm, 0.1 mW/cm^2) 1 min and 3 min. In some experiments the cells were pre-incubated with radachlorin (200 nM) during 5-10 minutes than washed three times and irradiated during 5 s, 30 s, 1 min, 3 min and 5 min.

The cells downloaded with BODIPY C11 [581/591] were also pre-incubated with 400 nM of radachlorin (5min) and irradiated.

Statistical analysis was performed with the aid of Origin 8 (Microcal Software Inc., Northampton, MA, USA) software. One Way Repeated Measures Anova test was used.

Results

Photodynamic effect of radachlorin induces calcium signal in neurons and astrocytes

Application of 200 nM radachlorin to primary co-culture of neurons and astrocytes had no effect on $[Ca^{2+}]_c$ of these cells. However, irradiation of radachlorin-loaded cells induced peak-like oscillations in neurons (Fig. 1 a, n=117 cells) and in much less degree in astrocytes (Fig. 1 b, n=126). Typical responses for neuron and astrocyte are shown in figure 1 c - d. Importantly, the number of cells with calcium response was directly dependent on the time of irradiation (Fig. 1 e – f).

Photo-induced calcium signal in neurons and astrocytes depends on intracellular Ca^{2+} stores

In order to identify the source of the calcium in photo-induced $[Ca^{2+}]_c$ changes in neurons and astrocytes we use calcium-free medium (HBSS without Ca^{2+} plus 0.5 mM EGTA). We found that irradiation of radacholrin-loaded cells in calcium free medium did not block $[Ca^{2+}]_c$ changes in both

neurons (Fig. 2 a, n = 169) and astrocytes (Fig. 2 b, n = 162). Thus, photo-induced calcium signal is independent of external calcium. Considering this, we empty the intracellular (reticular) calcium store by blocking the SERCA by thapsigargin (1 μM). Subsequent irradiation of the cells did not induce any changes in $[Ca^{2+}]_c$ neurons (Fig. 2 c, n = 108) and astrocytes (Fig. 2 d, n = 143). Thus, irradiation of radachlorin-loaded cells induces release of Ca^{2+} from ER.

Photodynamic effect of radachlorin leads to phospholipase C activation

Most common mechanism of the calcium release from ER in neurons and astrocytes is IP3 receptors. IP3 is produced by phospholipase C. We pre-incubate primary co-culture of neurons and astrocytes with specific inhibitor of phospholipase C – 5 μM U73122 and found that it completely blocked photo-induced calcium signal in neurons (Fig. 3 a, n = 20) and astrocytes (Fig. 3 b, n = 18). Thus, photodynamic effect of radachlorin induced activation of phospholipase C and IP3 dependent calcium signal in neurons and astrocytes. Irradiation of photosensitizers induced ROS production [15, 16]. In order to test if ROS is a trigger for activation of phospholipase C, we used water soluble analogue of vitamin E – Trolox (500 μM, 30 min of pre-incubation). Importantly, pretreatment with antioxidant completely block effect of radachlorin irradiation on $[Ca^{2+}]_c$ neurons (Fig. 4 a, n = 19) and astrocytes (Fig. 4 b, n = 19).

Photodynamic effect of radachlorin stimulates lipid peroxidation in neurons and astrocytes

Phospholipase C can be activated by overproduction of oxidized lipids [5, 6]. Using BODIPY C11 [581/591] as a fluorescent indicator for lipid peroxidation, we measured the photodynamic effect of radachlorin on the rate of the lipid peroxidation. We have found that 2 and 4 minutes photoactivation of radachlorin–loaded cells induced significant increase in the rate of lipid peroxidation (rate of BODIPY C11 [581/591] oxidation rose to 120±10% of basal rate for neurons, n=18, $p<0.05$; and to 280±70% of basal rate for astrocytes, n=28 $p<0.01$; Fig. 5 a - c). Thus, irradiation with radachlorin induces ROS that trigger lipid peroxidation. Oxidized lipids stimulate phospholipase C, which produces IP3 and initiates calcium release from ER to cytosol.

Discussion

High selectivity of malignant tissue destruction during PDT is achieved by relatively specific accumulation of photosensitizer in tumor cells. The irradiation of photosensitized cells leads to direct toxic effect, vascular damage and stimulation of immune reactions [17]. Since PDT is used for treatment of brain tumors its effect on healthy tissue should be taken into account. It was shown previously that PDT can stimulate cell death in healthy neurons and glial cells [18, 19].

Here we show that irradiation with radachlorin induce lipid peroxidation in primary co-culture of neurons and astrocytes. Irradiation can produce it acting directly to lipids or indirectly through production of ROS. Considering the effect of Trolox and production of ROS during photodynamic effect of chlorine

derivative photosensitizers [15, 16] we can suggest that photodynamic effect of radachlorin induces production of ROS that is trigger for subsequent lipid peroxidation.

Lipid peroxidation is a trigger for membrane instability and further toxicity by its byproduct [20]. Lower or moderate level of lipid peroxidation can induce activity of the number of phospholipases including phospholipase C [4, 21, 22]. In our experiments lipid peroxidation induced activation of phospholipase C that can be proven by inhibition with U73122. This activation play two important functions – removal of oxidized lipids and initiation of the calcium signal.

One of the interesting characteristics of the photodynamic effect of radachlorin on the calcium signal of neurons and astrocytes is that increase in the time of irradiation increases the number of cells with response, but not the amplitude of the signal (Fig. 1). It suggests that such mode of irradiation does not induce calcium signal of any other nature (such as permeabilization of the membranes). Difference in the higher affinity of the neurons to radachlorin irradiation and subsequent calcium signal compared to astrocytes can be explained by higher level of endogenous antioxidants (GSH) in glia compared to neurons [23].

Photo-induced calcium signal in neurons and astrocytes can play protective role: in stimulation of the number endogenous processes for cell protection or just for utilization of oxidized lipids by phospholipase C. However, irradiation of the radachlorin-loaded neurons in some of the brain areas can induce this signal that can interfere with regulatory signal in CNS.

Calcium signal is energy consuming process and prolonged and intensive elevation in $[Ca^{2+}]_c$ in combination with disruption in energy metabolism can induce energy deprivation and excitotoxicity [24, 25]. Considering the effect of PDT on the energy metabolism [26], activation of the calcium signal in neurons and astrocytes by photodynamic treatment with radachlorin can be potentially damaging for healthy tissue.

Conflict of Interest: The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1 Irradiation in presence of radachlorin induces increase in $[Ca^{2+}]_c$ neurons and astrocytes

Irradiation with radachlorin induce peak-like oscillations of $[Ca^{2+}]_c$ in neurons (a and b) and astrocytes (c and d). Each trace represents changes in the cytosolic Ca^{2+} in a single cell. The cells were pre-incubated with radachlorin (200 nM, 10 min) than washed and irradiated; during the experiment the concentration of radachlorin was increased several times. Longer time of irradiation results in increase of the number of cells (e – neurons, f - astrocytes) responded to photodynamic effect of radachlorin. Data on (e) and (f) panels are presented as the mean ± s.e.m. * - $p<0.05$.

Fig. 2 $[Ca^{2+}]_c$ changes in response to irradiation with radachlorin are dependent on intracellular Ca^{2+} stores in neurons and astrocytes

Removal of external Ca^{2+} (Ca^{2+}-free HBSS with 0.5 mM EDTA) does not abolish photo-induced Ca^{2+} responses in neurons (a) and astrocytes (c). Depletion of the intracellular Ca^{2+} pool by application of the inhibitor of ER Ca^{2+} pump, thapsigargin (1μM), abolishes the photo-induced Ca^{2+} signal in neurons (b) and astrocytes (d). Each trace represents changes in the cytosolic Ca^{2+} in a single cell. The cells were irradiated in presence of radachlorin (200 nM) during 1 and 3 min.

Fig. 3 Impact of inhibitor of phospholipase C activity on photo-induced Ca^{2+} signal in neurons and astrocytes

Pre-incubation with inhibitor of phospholipase C activity, U73122 (5 μM, 15 min), blocked changes in $[Ca^{2+}]_c$ in response to irradiation (1 and 3 min) in presence of 200 nM of radachlorin in neurons (a) and astrocytes (b). Each trace represents changes in the cytosolic Ca^{2+} in a single cell.

Fig. 4 Effect of water-soluble analogue of vitamin E, Trolox, on photo-induced Ca^{2+} signal in neurons and astrocytes

Pre-treatment with antioxidant, Trolox (500 μM, 30 min), that inhibits lipid peroxidation almost completely blocked Ca^{2+} changes in response to irradiation (1 and 3 min) with radachlorin (200 nM) in neurons (a) and astrocytes (b). Each trace represents changes in the cytosolic Ca^{2+} in a single cell.

Fig. 5 Effect of photodynamic treatment on lipid peroxidation in neurons and astrocytes

Irradiation with radachlorin (400 nM) induces increase in the rate of lipid peroxidation in both neurons (a and b) and astrocytes (c and d). Traces in panels (a) and (c) represent the mean ± s.e.m. fluorescence measured in neurons and astrocytes in a representative experiment.

Panels (b) and (d) represent increase in the rate of lipid peroxidation as a percentage of the basal rate of BODIPY C11 [581/591] oxidation.

The cells were pre-incubated with radachlorin (5 min) than irradiated two times for 2 minutes. * - $p<0.05$