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REVIEWS

Disruption of Calcium Homeostasis and Following Changes in Calcium Signaling in Neurons and Glial Cells in Response to Photodynamic Treatment

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Abstract—Photodynamic impact on neurons and glial cells, causing oxidative stress and ischemic damage, is accompanied by disruption of calcium homeostasis and activation or suppression of diverse calcium-dependent mechanisms, such as calcium pumps and channels, calcium-dependent signaling or executive proteins, and other signaling systems that interact with calcium pathway, like ion channels, pumps and exchangers, nitric oxide, glutamate, and others. The cascade of processes initiated by oxidative stress and ischemia in nervous tissue, includes both protective responses and apoptotic or necrotic cell death scenarios. This mini review surveys the publications on these processes and compares them with the data obtained in our laboratory on the model of photothrombotic stroke on rat brain in vivo and the model of photodynamic treatment on crayfish mechanoreceptor in vitro. These areas of research are driven by the need to find methods of emergency neuroprotection in ischemic stroke and to improve the accuracy and efficiency of photodynamic therapy of tumors with minimized damage to benign tissues. A proteomic study of the penumbra region in the photothrombotic stroke model revealed changes in the expression of a number of calcium-dependent proteins associated with impaired calcium homeostasis and having either a protective or damaging tendency. Inhibitory analysis of the effects of photo-oxidative stress on the crayfish stretch receptor model revealed the involvement of a number of proteins in the calcium-dependent pathway in neuronal or glial cell death or survival. In this paper, these data are analyzed and summarized to identify promising directions for further research.

Keywords: calcium signaling, glutamate signaling, neuron, glia, photothrombotic stroke, photodynamic therapy

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INTRODUCTION

The studies conducted in our laboratory have overlaps with the works of B.I. Khodorov on the molecular and cellular bases of neurotoxicity, including disruption of calcium homeostasis.

Calcium, as a universal secondary messenger in the signal transduction system, integrates a number of signaling pathways controlling various functions and cell death. An increase in cytosolic Ca^{2+} concentration to 10^{-4}–10^{-3} M triggers a necrotic or apoptotic scenario of cell death. Different systems of calcium homeostasis maintenance work simultaneously in cells: sodium–calcium and other exchangers, passive calcium channels with different activation mechanisms, active calcium pumps, specific mitochondrial mechanisms of Ca^{2+} entry and release, store-dependent mechanisms, and calcium-dependent proteins.

The central role of Ca^{2+} in physiological and pathological processes particularly necessitates the study of their role in the processes induced by oxidative stress. This review will consider the studies of calcium homeostasis disruption occurring in photothrombotic brain stroke and photodynamically induced oxidative stress of neurons and adjacent glia.

Abbreviations: PTI, photothrombotic infarction; PD, photodynamic; ROS, reactive oxygen species; ER, endoplasmic reticulum; TRP, transient receptor potential; ASICs, acid-sensitive ion channels; CNS, central nervous system; CaMK, calmodulin-dependent kinase; PKC, protein kinase C; PLC, phospholipase C; IP3, inositol-1,4,5-triphosphate; APP, β-amyloid precursor protein; Aβ, β-amyloid; CSR, crayfish stretch receptor; MRN, mechanoreceptor neuron; LP, lipid peroxidation; NAAG, N-acetyl-aspartyl-glutamate; GCP II, glutamate carboxypeptitase II.

PHOTOTHROMBOTIC STROKE AND CALCIUM HOMEOSTASIS

Stroke is a multistage process in which cell damage propagates from the focus of infarction to the surrounding tissues, resulting in the formation of a transition zone, the so-called ischemic penumbra, where cell death develops slower, within a few hours [1, 2].

Calcium ions are universal intracellular messengers both during ischemic damage and in the postischemic period. In particular, they play a key role in the mechanism of excitotoxicity, activation of proteolytic enzymes, initiation of necrosis and apoptosis, propagation of damaging processes beyond the infarct core and penumbra formation. Previous studies have shown that elevated Ca^{2+} levels affect infarct size and determine the outcome and recurrence of ischemic stroke [3]. Such a multifaceted role of Ca^{2+} explains the need for further studies on the role of calcium in the initiation of cell death after ischemic attack.

According to the current paradigm, oxygen and glucose deficiency in the ischemic focus causes ATP depletion, formation of reactive oxygen species (ROS), oxidative membrane damage, loss of ionic gradients, Ca^{2+} influx, K^+ release, oxidative stress, and tissue edema. K^+-mediated depolarization promotes the opening of NMDA channels in neighboring neurons, additional Ca^{2+} influx, and K^+ and glutamate release [4, 5]. Such a self-developing excitotoxic process leads to the propagation of damage beyond the infarction focus [6, 7].

Intracellular Ca^{2+} induces mitochondrial dysfunction, ROS formation, and nitric oxide (NO) production. Ca^{2+}-activated neuronal NO synthase, which is often spatially linked to NMDA receptors, produces nitric oxide, which reacts with O^{2-} forming the potent oxidant peroxynitrite ($ONOO^-$). In addition, cytosolic Ca^{2+} activates various hydrolytic enzymes, such as proteinases, lipases, and nucleases, which degrade cellular components, leading to cell death [8]. In particular, Ca^{2+}-activated calpain and cathepsins play an essential role in neurodegeneration. Ca^{2+}-mediated signaling pathways promote various transcription factors such as NF-kB, AP-1, CREB, STAT, etc., which regulate the expression of proteins involved in cell survival or death [9–11]. Excessive intracellular Ca^{2+} can enter neuronal nuclei and activate the expression of specific genes depending on the influx pathway. Ca^{2+} influx through NMDA synaptic receptors can induce a calcium wave that propagates to the nucleus. Nuclear calcium promotes the CaMKIV/CREB pathway, which induces the expression of genes involved in the regulation of physiological functions: metabolism, synaptic transmission, and cell survival. In contrast, hyperactivation of extrasynaptic NMDA receptors under stress or ischemia induces pathogenic Ca^{2+} overload and expression of genes related to apoptosis and neurodegeneration. In this case, CREB can be functionally inactivated by rapid dephosphorylation and subsequent degradation. Activation of these receptors causes accumulation of histone deacetylases HDAC4 and HDAC5 in the nucleus, which repress transcription. It also promotes the accumulation of the proapoptotic transcription factor FOXO3A in the nucleus. In addition, Ca^{2+} overload rapidly decreases the mitochondrial membrane potential [9, 10, 12].

In our laboratory we studied the role of Ca^{2+} in the cellular and molecular mechanisms of focal photothrombotic infarction (PTI) in the cerebral cortex of rats and mice induced by local photodynamic exposure [9–11]. The photodynamic effect was based on photoexcitation of Rose bengal dye in stained cells followed by generation of singlet oxygen and other ROS, oxidative stress and finally cell necrosis or apoptosis. Photo exposure causes local oxidative damage to the vascular endothelium, followed by platelet aggregation and microvascular occlusion.

Already during the first 10 min after PTI, an abrupt increase in cytosolic Ca^{2+} levels is noticed [6, 13]. The following mechanisms of intracellular Ca^{2+} increase are distinguished: influx through NMDA receptors, potential-dependent Ca^{2+} channels, [12, 14]; release from mitochondria and endoplasmic reticulum (ER); inhibition of Ca^{2+}-ATPases in ER and plasma membrane and disruption of Na^+/Ca^{2+} exchange [7, 12]; long-term activation of transient receptor potential (TRP) channel proteins [12, 15], non-selective cation-dependent channels that can be activated regardless of membrane depolarization. In the stroke core, cell death occurs immediately, but in the penumbra many cells strive to protect themselves via various internal mechanisms, including Ca^{2+} exchange regulation. A decrease in extracellular Ca^{2+} and Mg^{2+} leads to TRPM7 activation. This decrease also activates acid-sensitive ion channels (ASICs), which are activated during ischemia due to lactic acid and proton production. TRPM7, TRPM2 and ASICs have been shown to contribute to delayed cell death in central nervous system (CNS) in stroke [15, 16].

It has been shown that under ischemic conditions glutamate binding with the NMDAR2a receptor evokes Na^+ and Ca^{2+} influx into neurons and K^+ release, which depolarizes neighboring cells and mediates the propagation of excitotoxicity. The Ca^{2+}-dependent excitotoxic response leads to apoptosis. In penumbra after PTI, the observed overexpression of NMDAR2a and glutamate decarboxylase (GAD65/67), responsible for the transformation of *L*-glutamate into gamma-aminobutyric acid (GABA), was another pro-apoptotic effect associated with glutamate metabolism [11]. This effect is consistent with the observation that the embryonic variant of GAD67 splicing induces apoptosis in the adult brain in stroke [17–19].

The Ca^{2+}/calmodulin complex regulates numerous functions, such as neurotransmitter production and release, cytoskeleton remodeling, axonal transport, gene expression, cell survival, learning, and memory. Calmodulin and calmodulin-dependent kinases, CaMKII and CaMKIV, are widely distributed in the mammalian brain. Our studies showed that their levels in ischemic penumbra were elevated after PTI.

CaMKII-mediated neuroprotection is associated with phosphorylation and inhibition of some proapoptotic proteins, such as NO synthase and GSK-3β. On the other hand, CaMKII phosphorylates glutamate receptors NMDA and AMPA. This increases Ca^{2+} influx and exacerbates excitotoxicity. CaMKII inhibitors protect the brain from ischemic damage. CaMKIV inhibits excitotoxicity by activating the antiapoptotic pathway PI3K/Akt and phosphorylation of CREB, which mediates the expression of several antiapoptotic genes [11].

Another Ca^{2+}-dependent protein, calreticulin, regulates calcium homeostasis and controls protein folding in the ER. It modulates the expression and activity of p53 and thus regulates apoptosis. Calreticulin has been shown to inhibit neuronal apoptosis in penumbra in the early stages of stroke [7]. Thus, the activation of calreticulin in the penumbra, that we observed via proteomic analysis 4 h after PTI may play a protective role [9].

At the same time, the level of some signaling proteins in PTI-induced penumbra significantly decreased. It is possible that massive Ca^{2+} influx into penumbra cells during brain damage and edema causes proteolysis and decreased expression of Ca^{2+}-dependent proteins. For example, the levels of Ca^{2+}-dependent signaling proteins such as phospholipase Cγ (PLCγ) and protein kinase C (PKC) were decreased in PTI-induced penumbra. PLCγ upon plasma membrane damage or tyrosine kinase receptor activation cleaves membrane phospholipids and releases inositol-1,4,5-triphosphate (IP3), which promotes Ca^{2+} release from ER, and diacylglycerol, which activates PKC [11]. Ca^{2+}-activated PKC and its isoforms α, γ, δ, ε, η, and μ control multiple cell functions, such as homeostasis regulation, synaptic processes, proliferation, apoptosis, and brain responses to ischemic damage [9, 10]. When studying the expression of PKC and its isoforms using signaling and neuronal microarrays [9], we obtained contradictory results. The use of neuronal microarrays showed more than a 4–6-fold decrease in the total PKC level in the penumbra 1 and 4 h after PTI. These results are consistent with a rapid loss of total PKC levels and its activity after ischemic brain injury due to, probably, its degradation. PKC inhibitors protected neurons from postischemic and excitotoxic damage, indicating the involvement of PKC in neurodegeneration. Therefore, the observed decrease in PKC levels could correspond to a decrease in nerve cell damage in penumbra. The expression of its isoform PKCβ1 in penumbra also significantly decreased by a factor of 1.65–2 during the whole period studied from 1 to 24 h after PTI, but the level of another isoform PKCβ2, on the contrary, increased after 4 h. However, using signaling microarrays [11], we observed an increase in PKCβ and PKCα levels by 34–36% and 31–54%, respectively, 4–24 h after PTI. It can be assumed that there is more PKCβ2 isoform than PKCβ1 in penumbra tissue. Therefore, it was the PKCβ2 isoform that decisively contributed to the increase in total PKCβ levels. The levels of both protein kinases, PKCα and PKCβ, in the rat brain also increased after incomplete global ischemia, which the authors associate with increased permeability of the blood-brain barrier. Protein kinase Cμ (PKCμ, also called PKD) acts as a neuroprotector during cerebral ischemia. It phosphorylates the chaperone Hsp27, which suppresses neuronal apoptosis mediated by the ASK1/JNK cascade. We observed PKCμ (PKD) activation in PTI-induced penumbra, which generally agrees with literature data [9].

The levels of Ca^{2+}-binding protein S-100 and its β-chain (S-100β) decreased more than 2-fold in penumbra 1–24 h after PTI. S-100β protein is specific for nervous tissue, in particular for glial cells. In the nanomolar range, it demonstrates neuroprotective properties, but at micromolar concentrations it can induce apoptosis. The appearance of S-100 and S-100β in blood serum may be a marker of such neuropathologies as acute stroke, neurotrauma, brain tumor, and neurodegenerative diseases [9–11]. Ca^{2+} also indirectly controls gene expression through Ca^{2+}-dependent interaction between calmodulin and S-100 with dimeric transcription factors, which prevents these factors from binding to DNA [9].

One and 4 h after PTI, the level of protein VILIP1 in penumbra decreased by 66–71%. This Ca^{2+}-binding protein serves as one of the Ca^{2+} sensors in the cell. It is wide-spread in the brain. VILIP1 influences various intracellular signaling cascades, including cyclic nucleotide systems and MAP-kinase pathway. When the cytosolic Ca^{2+} concentration increases, VILIP1 binds to the cell membrane and regulates vesicular trafficking and recirculation of receptors and ion channels. After stroke, VILIP1 is released from damaged nerve cells into cerebrospinal fluid and blood serum, being a marker of stroke [9]. The Ca^{2+} sensor synaptotagmin is responsible for the fusion of synaptic vesicles with the synaptic membrane and neurotransmitter release. PTI exposure resulted in suppression of its expression. This was consistent with the ultrastructural evidence of destruction and disorganization of synaptic vesicles. In rats, synaptotagmin mRNA was suppressed during early recirculation after focal cerebral ischemia [9, 11].

Proteomic analysis showed increased expression of β-amyloid precursor protein (APP) in PTI-induced penumbra during the first 24 h after exposure [11]. Our results are consistent with the data on APP activation after cerebral ischemia, which is considered as an adaptive and neuroprotective response [20]. Protective effects of APP or its soluble fragments (APPsα) have been shown on different animal models of ischemic stroke. The main mechanisms of this protective effect include APP-mediated regulation of calcium homeostasis via NMDA receptors, as well as voltage-

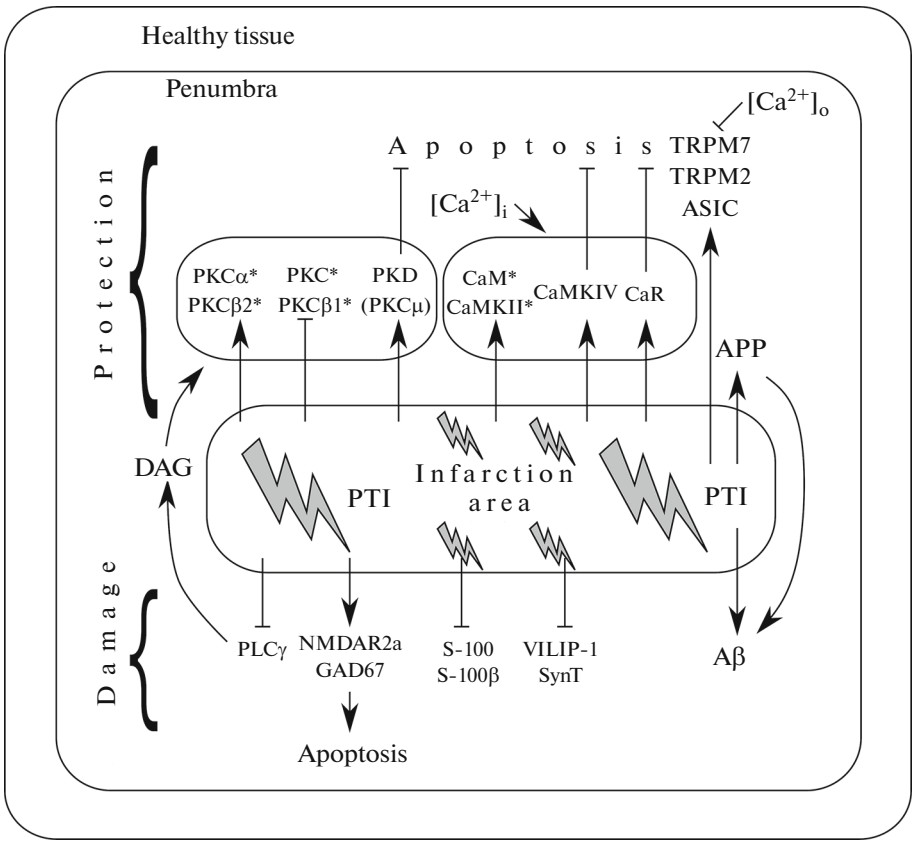

Fig. 1. Protective and damaging effects of photothrombotic stroke on the expression of calcium-dependent proteins in penumbra. *Arrows with sharp ends* indicate promotion effects, *arrows with blunt ends* indicate suppressive effects. PTI, photothrombotic infarction; PKC, protein kinase C; CaM, calmodulin; CaMK, calmodulin kinase; CaR, calreticulin; APP, amyloid precursor protein; Aβ, β-amyloid; DAG, diacylglycerol; PLC, phospholipase C; SynT, synaptotagmin; $[Ca^{2+}]_o$, extracellular calcium concentration; $[Ca^{2+}]_i$, intracellular calcium concentration. Asterisks (*) indicate conflicting data on the protective/damaging nature of the effect. For the rest of the notations, see the text.

dependent calcium channels (VGCC) and intracellular calcium stores [12, 20]. Studies on knockout mice have shown that under ischemic conditions, mutations of presenilin-1 (PS1), which is involved in APP proteolysis, lead to disruption of cellular calcium homeostasis in neurons, which is associated with calcium release from ER, and thus increase their vulnerability to ischemic damage [20]. Our studies have also demonstrated increased levels of β-amyloid [9], a fragment of amyloid peptide (Aβ) that stimulates Ca^{2+} influx into the cell and thus has neurotoxicity. The mechanisms of APP-mediated neuroprotection via the regulation of cellular calcium levels by blocking L-type calcium channels (LTCC) and NMDA receptors determine new therapeutic targets in anti-stroke therapy [20].

The data on the effect of photothrombotic ischemia on the expression of calcium-related functional proteins are summarized in Fig. 1. Since Ca^{2+} is one of the key factors of ischemic cell damage, various Ca^{2+}-channel blockers as well as inhibitors of Ca^{2+}-binding proteins have been tested in numerous searches for neuroprotectors. However, no effective neuroprotectors have yet been found. Even compounds that showed positive effects in experimental animals were not effective in humans or caused undesirable side effects [1, 2, 8]. Data on the effect of Ca^{2+} on the processes of cell survival and death in ischemic stroke are still incomplete and sometimes contradictory, but they are sufficient for understanding the importance of this ion in cell survival and death. Therefore, in-depth, comprehensive study of Ca^{2+}-involving molecular mechanisms of neurodegeneration and neuroprotection in penumbra tissue is necessary to develop new approaches to treat the consequences of stroke.

PHOTODYNAMIC TREATMENT AND CALCIUM HOMEOSTASIS IN NEURONS AND GLIAL CELLS

Photodynamic therapy is a medical method in which light radiation destroys pathologically altered tissue (e.g., tumor tissue) selectively stained with special photosensitizing compositions. The photodynamic (PD) effect is based on the photochemical

transformation of molecules in an electron-excited state, during which the formation of ROS occurs, among which singlet oxygen is the most important, which can damage only the biostructures that are in the immediate vicinity of the photosensitizer molecules. In the CNS, tumors can form only proliferation-capable glia, but not neurons. Various signaling pathways, including calcium-dependent mechanisms involved in the cellular response of neurons and glia to PD exposure in the isolated abdominal mechanoreceptor of freshwater crayfish have been studied at the fundamental level in our laboratory [21, 22], as well as together with the laboratory of A.Yu. Abramov on primary cultures of rat neurons and astrocytes.

Involvement of Calcium Signalling Pathway in Photodynamic Damage of Neurons and Glial Cells

The involvement of Ca^{2+} and PKC in the electrophysiological response of the neuron to photodynamic damage was studied with the widely clinically used drug Photosense (hydroxyaluminum trisulfophthalocyanine), on an isolated mechanoreceptor of freshwater crayfish. Activation of PKC by 12-O-tetradecanoylphorbol-13-acetate (TPA) and increase of cytosolic Ca^{2+} concentration by ionomycin and thapsigargin accelerated PD-induced termination of firing activity. In contrast, PKC inhibition by staurosporine, hypericin, and cheleritrin prolonged the time of functional activity. This shows the involvement of Ca^{2+} and PKC in PD-induced inactivation and subsequent death of the neuron [23]. PD exposure not only leads to cessation of firing and neuronal necrosis, but also causes necrosis and apoptosis, as well as proliferation of the surrounding glia. The involvement of calcium-dependent signaling pathway proteins, calmodulin, CaMKII and PKC, in this effect was studied using their specific inhibitors. It was found that calmodulin and CaMKII are involved in PD-induced neuron and glia necrosis, while PKC protects glia from apoptosis and necrosis under PD exposure [24].

Calcium Response to Photodynamic Effects of Radachlorin

In the crayfish stretch receptor (CSR) model, the promising photosensitizer Radachlorin accumulated predominantly in the glial sheath and relatively poorly penetrated into the body and axon of the mechanoreceptor neuron (MRN). Photoactivated Radachlorine reduced the duration of neuron firing and induced necrosis and apoptosis of satellite glia at concentrations of 10^{-9} M and lower [25]. The PD effect of Radachlorin was used to detect the effects of photoinduced oxidative stress on calcium signaling and lipid peroxidation (LP) in primary cultures of neurons and astrocytes using in vivo cellular imaging. Irradiation in the presence of 200 nM Radachlorin induced calcium signaling in neurons and astrocytes, which depends on intracellular calcium stores because it can be blocked by ER depletion and by inhibition of the Ca^{2+}-ATPase ER by thapsigargin. The calcium response was also blocked by PLC inhibition. At the same time, Radachlorin induced LP in neurons and astrocytes. It can be assumed that PTI-induced LP leads to PLC activation, which leads to IP3 production, which, in turn, causes Ca^{2+} release from ER into cytosol via IP3 receptors [26]. It was also found that PD exposure of cells with Radachlorin leads to mitochondrial depolarization in both neurons and astrocytes and reduces the level of mitochondrial NADH [27].

Calcium-Dependent Regulation of Nitric Oxide Production under Photodynamic Exposure

Nitric oxide (NO) regulates many physiological and pathophysiological processes, including neurotransmission, vasodilation, stress response, etc. It has a protective effect in apoptosis and LP as an antioxidant. Photodynamic exposure with Photosense in the CSR model induced NO generation in glial cells and dendrites and, to a lesser extent, in the soma and axon of the neuron. Using cytosolic calcium level modulators, Ca^{2+} and NF-kB were shown to regulate NO generation in photosensitized neurons and glia. NO production was promoted by a fourfold increase in extracellular Ca^{2+} concentration, ionomycin, and inhibition of the ER Ca^{2+} ATPase. In contrast, blocking of plasma membrane calcium channels decreased NO production. Apparently, Ca^{2+}-dependent neuronal NO synthase is involved, along with Ca^{2+}-independent inducible NO synthase, in NO production in neurons and glial cells of freshwater crayfish [28].

Action of Glutamate under Photodynamic Exposure

Ionotropic glutamate receptors (NMDA in particular) are ion channels, particularly involved in Ca^{2+} transmission. Metabotropic glutamate receptors are involved in the regulation of voltage-dependent calcium channels and ER IP3 receptors. Addition of glutamate enhanced the pro-necrotic effect of PD exposure on neurons and glia, but reduced PD-induced apoptosis of glia. Crayfish neurons release N-acetyl-aspartyl-glutamate (NAAG), which is cleaved by GCP II (glutamate carboxypeptitase II) in the extracellular space to form glutamate. NAAG also suppressed PD-induced apoptosis. Inhibition of GCP II, on the other hand, promoted glia apoptosis. Inhibition of metabotropic glutamate receptors, but not NMDA, reduced PD-induced glia apoptosis. Thus, glutamate released via GCP II protects glia from apoptosis, apparently via metabotropic but not ionotropic receptors [29]. The results obtained are illustrated in Fig. 2.

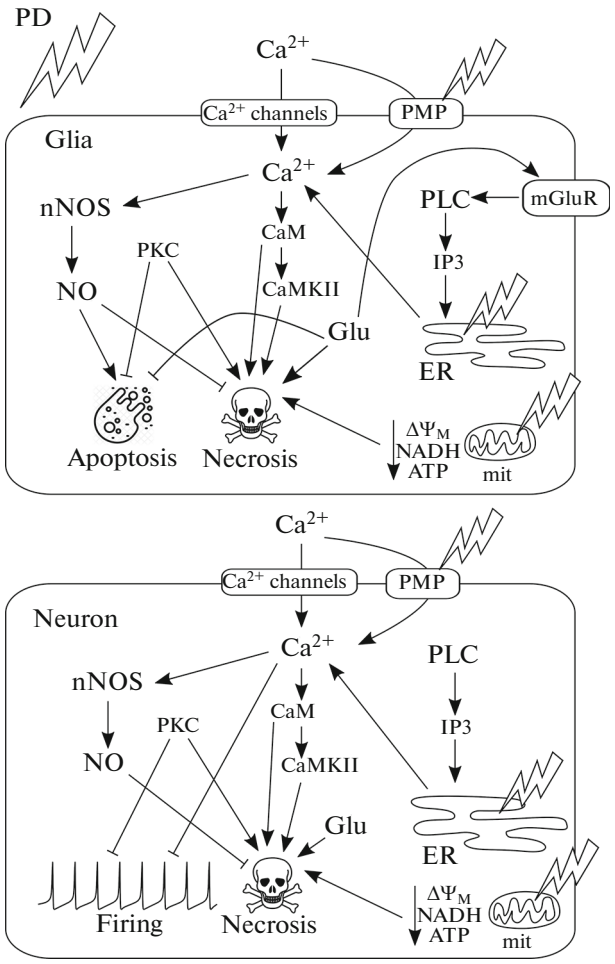

Fig. 2. Involvement of Ca^{2+}-dependent pathways, including NO- and glutamate signaling, in the responses of neurons and glial cells to photodynamic treatment. Potential targets of PD exposure are indicated by *lightning arrows*. *Arrows with sharp ends* indicate stimulatory effects, *arrows with blunt ends* indicate suppressive effects. Abbreviations: mit, mitochondria; ER, endoplasmic reticulum; CaM, calmodulin; CaMKII, calmodulin-dependent kinase II; nNOS, neuronal NO synthase; IP3, inositol triphosphate; PKC, protein kinase C; PLC, phospholipase C; PMP, plasma membrane pores; Glu, glutamate; mGluR, glutamate metabotropic receptors; $\Delta\Psi_M$, mitochondrial potential.

CONCLUSIONS

Disruption of calcium homeostasis in neurons and glial cells can be triggered by various factors, including photothrombotic stroke-induced ischemia and photodynamic damage-induced oxidative stress. Calcium ions, in turn, mediate various mechanisms of the cellular response to damage, aimed both at protection and degeneration of cells. Central to these processes is the concentration of calcium in the cytosol, which is maintained at low levels under physiological conditions but increases by several orders of magnitude under pathological conditions and often triggers irreversible signaling cascades leading to cell death. A CSR model study also confirmed the anti-apoptotic activity of glutamate, presumably related to metabotropic receptors, and the calcium-dependent increase in nitric oxide production under oxidative stress on neuronal physiological activity and neuronal and glial cell survival. In combination with data obtained in other models, these results contribute to the fundamental basis for further development of neuroprotection methods and tumor therapies.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

This article does not describe any studies involving humans or animals as subjects.

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