A Complex Neuroprotective Effect of Alpha-2-Adrenergic Receptor Agonists in a Model of Cerebral Ischemia—Reoxygenation In Vitro

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Abstract—The mechanism of the neuroprotective action of α_2-adrenergic receptor agonists in a model of oxygen-glucose deprivation (OGD) in vitro was investigated. Using fluorescent microscopy, immunostaining, inhibitor analysis, and real-time PCR analysis, we demonstrated that OGD evokes a biphasic $[Ca^{2+}]_i$ increase in all cells. Initial Ca^{2+} impulse in astrocytes and Ca^{2+} oscillations in neurons were followed by a slower global increase of $[Ca^{2+}]_i$ in both cell populations. Accumulation of pro-inflammatory factors, such as IL1b and TNFα, was observed during 40-min OGD. It was established that reoxygenation is followed by hyperexcitation of neurons, caspase-3 activation, and subsequent cell death. We showed that a 24-h pretreatment of cell cultures with selective α_2-adrenergic receptor agonists guanfacine and UK-14,304 abolished the global $[Ca^{2+}]_i$ increase in astrocytes and neurons but did not suppress the first phase of the OGD-induced Ca^{2+} impulse in astrocytes. In addition, the number of dead cells after OGD was decreased in cell cultures pretreated with the α_2-agonists. Guanfacine inhibited caspase-3 activation and suppressed apoptosis in our experiments. In particular, the expression of antiapoptotic genes *Stat3* and *Bcl-2* was enhanced after the pretreatment with guanfacine. On the contrary, the expression of proapoptotic genes (*Socs-3*, *p53*, *fas,* and *Ikk*) was decreased. Moreover, application of guanfacine evoked Ca^{2+} response in astrocytes and led to Ca^{2+}-mediated ATP release and this way suppressed hyperexcitation of the neurons. Thus, activation of astrocytes and Ca^{2+}-mediated ATP release possibly contribute to the complex neuroprotective effects of the α_2-adrenergic receptor agonists.

Keywords: neurons, astrocytes, calcium ions, apoptosis, adrenergic receptors, neuroprotectors, ischemia, hyperexcitation

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INTRODUCTION

In stroke, neurons are damaged and die due to a rapid secretion of excitatory amino acids, primarily glutamate, and its accumulation in the extracellular space, the disruption of transmembrane gradients and the associated acidosis [1]. Excitotoxic glutamate concentrations cause an increase in the concentration of Ca^{2+} ions in the cytosol of neurons [2], leading to increased production of free radicals, activation of lipid peroxidases, proteases, phospholipases, development of oxidative stress and accumulation of free fatty acids in the cytosol [3, 4]. All these processes lead to the destruction of cell membrane structures and death. The depolarization of neurons and the Ca^{2+} entry into the cytosol through voltage-gated Ca^{2+} channels leads to the release of other neurotransmitters, in particular norepinephrine. There is evidence that the release of norepinephrine observed during ischemia may contribute to post-ischemic brain damage [5, 6]. However, norepinephrine, activating α_2-adrenergic receptors, can play a protective role in ischemia through inhibition of adenylate cyclase and suppression of excitatory neurotransmitters secretion by neurons [7].

Adrenergic receptors are widely expressed in mammalian tissues and belong to receptors associated with G proteins. They are divided into several types (α_1, α_2, β_1, β_2, β_3) differing in the affinity to agonists and represented by several subtypes. Of greatest interest are α_2 adrenoreceptors, expressed in astrocytes, glutamatergic and GABAergic neurons, which respond by increasing $[Ca^{2+}]_i$ to the application of a selective agonist, guanfacine [8−10]. However, in experiments performed on rat hippocampal culture, α_2-adrenoreceptor agonists at concentrations that have a neuroprotective

effect, increased $[Ca^{2+}]_i$ only in astrocytes, which is apparently due to the increased expression level of these receptors in glial cells [11].

All subtypes of α_2 adrenoreceptor activate Gi proteins, which, on the one hand, inhibit the activity of adenylate cyclase and protein kinase A (PKA) through the α-subunit of Gi [12]. PKA regulates the activity of the kinase phosphorylase, MAP-2, AMPA receptors, and L-type Ca^{2+} channels [13, 14]. On the other hand, $\beta\gamma$-subunit of Gi can participate in the mobilization of calcium from intracellular stores through the activation of phospholipase C [15], in glial cells in particular [16].

The potential for use of α_2-agonists for treatment of various pathologies of the nervous system [17], including cerebral ischemia [7], has been studied for a long time. Moreover, some of the agonists are quite successfully used in clinical practice [18]. However, there are significant limitations due to the lack of knowledge about the intracellular mechanisms of action of α_2-adrenoreceptor agonists on brain cells, as well as side effects (hypotension and bradycardia) produced by α_2-adrenoreceptor activators used for prophylactic purposes. Molecular and cellular processes activated by α_2-adrenergic receptors in order to protect the brain from ischemic damage need to be studied in detail. In this work, we studied the relationships between neuroprotective effects of selective α_2-adrenoreceptor agonists, guanfacine and UK-14,304, and changes in the cytosolic calcium concentration, release of gliotransmitters, expression of protective genes, and suppression of apoptosis and inflammation that occur during ischemia and reoxygenation.

MATERIALS AND METHODS

Hippocampal cell culture. A mixed neuroglial hippocampal cell culture was obtained from newborn (P1−3) linear Spraque Dawley rats. After decapitation, the hippocampus was removed and transferred to ice-cold Hanks solution. The tissue was minced with scissors, placed in the Versene solution with 0.2% trypsin, and incubated for 10 min at 37°C in a thermoshaker at 600 rpm. The enzyme-treated pieces of tissue were washed three times with neurobasal medium, carefully pipetted, and centrifuged (2 min at 300 g). Then the supernatant was removed, the cells were resuspended in neurobasal medium containing glutamine (0.5 mM), Supplement B27 (2%), and gentamicin (15 µg/mL). The suspension was set into glass cylinders with ground ends with an internal diameter of 6 mm, standing on round cover glasses with a diameter of 25 mm (VWR International) coated with polyethylenimine, and placed in 35 mm Petri dishes (Greiner). One hundred µL of the cell suspension was added to each cylinder and left for 2 h to attach in a CO_2 incubator at 37°C. After that the cylinders were removed, and the volume of the culture medium was adjusted to 1.8 mL. Every 3 days, 2/3 of the volume of the culture medium was replaced with fresh. Experiments were performed on cultures at the age of 10 days in vitro (10 DIV).

Cell staining. The concentration of calcium ions in the cytoplasm ($[Ca^{2+}]_i$) was estimated using a two-wave Fura-2 probe in accordance with a well-known method [19]. For staining of hippocampal cells, Fura-2 AM ether was used at a final concentration of 4 µM in Hanks solution containing (in mM): 156 NaCl, 3 KCl, 1 $MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.4. Freshly prepared dye solution (200 µL) was added to each cover glass with cell culture and incubated in a thermostat for 40 min at 37°C. After that the culture was washed with Hanks solution and incubated for 10−15 min to complete the deesterification of the dye.

Fluorescence measurements. Changes in the level of cytosolic calcium in the cells were recorded using the Cell Observer image analysis system (Carl Zeiss, Germany) based on an Axiovert 200M inverted microscope equipped with an AxioCam HSm monochrome CCD camera and a Ludl MAc5000 high-speed change filter system. A Plan-Neofluar 10×/0.3 lens was used. An illuminator with an HBO 103W/2 mercury lamp was used as a source of fluorescence excitation. For the excitation and recording of Fura-2 fluorescence, a Filter set 21HE light filter set (Carl Zeiss, Germany) with BP340/30 and BP387/15 excitation filters, an FT409 splitter, and a BP510/90 emission filter were employed. To measure fluorescence intensity, a round cover glass with a cell culture was mounted in a special measuring chamber. The volume of medium in the chamber was 0.5 mL. Application of the reagents and washing were carried out by replacing the medium with a tenfold volume by a perfusion system at a rate of 15 mL/min. The measurements were carried out at 28°C. A series of images were obtained with an interval of 1 frame in 3 s. To identify neurons in the experiment, 35 mM KCl was applied to the cells for 30 s; this short-term test application causes depolarization and an increase in the concentration of cytosolic calcium in excitable cells, neurons in our case. Ca^{2+} response in neurons evoked by the application of high concentrations of K^+ is characterized by a rapid rise and a slow decline [20]. This response is absent in glial cells [21]. For identification of glial cells, astrocytes in particular, a short-term application of ATP was used. Astrocytes respond to ATP with a fast biphasic Ca^{2+} response with a slow decline [22]; in hippocampal neurons ATP does not evoke such a response [20].

The obtained time series of two-channel images (excitation wavelengths, 340 and 380 nm) were processed in the ImageJ program with a software module Time series analyzer. The calcium response amplitudes of single cells were evaluated as the ratio of Fura-2 fluorescence signals upon excitation at 340 and 380 nm. Plotting and statistical analysis was performed using

Origin 8.5. The results are presented either as single cell signals typical of neurons and astrocytes or as an average cell signal in the field of view ± standard error (SE).

Analysis of cell survival. The number of dead cells in the same culture area before and after oxygen-glucose deprivation was determined by staining cultures with propidium iodide (1 μM). Since propidium iodide is able to stain both neurons and glial cells, neuron identification was performed by application of 35 mM potassium chloride prior to oxygen-glucose deprivation. In response to the addition of potassium chloride neurons generate a fast transient calcium signal. The form of calcium signal upon oxygen-glucose deprivation (the presence or absence of a global increase in $[Ca^{2+}]_i$ during OGD) was an additional parameter for assessing cell viability.

To study the effects of α_2-adrenoreceptor agonists on OGD-induced initiation of apoptosis and necrosis processes, double staining with propidium iodide and fluorescent substrate of caspase-3, NucView488, was used. For this purpose, cultures were loaded for 1 h with NucView488 (final concentration 2 μM) before the experiments. The cultures were then subjected to a 40-min oxygen-glucose deprivation and after that stained with propidium iodide (1 μM). The dye fluorescence was recorded using an image analysis system based on the Axiovert 200M inverted fluorescence microscope equipped with a Hamamatsu ORCA-Flash 2.8 high-speed monochrome CCD camera. A Lambda DG-4 Plus illuminator (Sutter Instruments, USA) with a high-pressure mercury lamp was used. For excitation and registration of fluorescence emission, the following sets of light filters were used: Filter Set 10 with excitation filter BP 450-490, beam splitter FT510, emission filter BP 515−565, and Filter Set 20 with excitation filter BP 546/12, beam splitter FT560, filter emissions of BP 575−640. Five independent regions of each cover glass with cell culture were analyzed. Each experimental series consisted of at least three repeats. All results are presented as mean ± standard error (SE).

ImageJ and Origin 8.5 software were used for data analysis and graph plotting. Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad Software, La Jolla, USA). The statistical significance of the differences between the experimental groups was assessed by the analysis of variance (ANOVA) using the Tuki−Kramer test. Differences were considered significant at $p < 0.001$.

Immunocytochemical staining of hippocampal cells. TNFα in cells was detected using immunocytochemical staining. Cells were fixed for 4 min with 4% solution of paraformaldehyde in PBS with the addition of 0.25% glutaraldehyde. This was followed by three 5-min washes of the cells with an ice-cold PBS solution. Glutaraldehyde was added to fixative solution to prevent excessive leakage of TNFα from cells during the permeabilization period. For permeabilization, cells were incubated for 15 min in 0.1% Triton X-100 solution. Blocking of non-specific antibody binding was performed using a 10% donkey serum solution in PBS. Cells were incubated in blocking solution for 30 min at room temperature.

Primary antibodies were loaded for 12 h at 4°C. Polyclonal anti-TNFα goat antibodies (Santa Cruz Biotechnology, USA) dissolved in 1% donkey serum in a 1 : 500 ratio were used as primary antibodies. After incubation with primary antibodies, the cells were washed three times with PBS solution with 5-min intervals. Then the cells were loaded with secondary antibodies − polyclonal donkey antibodies to goat antibodies conjugated with the Alexa Fluor 488 dye (Abcam, United Kingdom). Incubation with secondary antibodies was performed at room temperature in the dark for 90 min. Secondary antibodies were dissolved in PBS at a ratio of 1 : 200. The fluorescence of antibodies was visualized using a Leica TCS SP5 inverted laser scanning confocal microscope (Leica, Germany). An argon laser with a band of 488 nm was used to excite the fluorescence. The emission was recorded in the range of 505−565 nm.

Simulation of ischemia-like conditions in vitro. To create an oxygen-glucose deprivation (OGD) in Hanks solution, glucose was replaced with an equivalent amount of sucrose, and dissolved oxygen was replaced by blowing the solution with argon in a special hermetic system. The oxygen level in the ischemic medium, measured with a Clark electrode, was 30−40 mm Hg. The ischemic medium was delivered to the experimental chamber with cells for 40 min using a perfusion system with a constant inert gas blow, which made it possible to avoid contact with atmospheric oxygen. The effect of oxygen-glucose deprivation on cells was assessed by the amplitude and pattern of Ca^{2+} signals.

Quinacrine staining. To visualize ATP-containing vesicles, astrocytes were stained by incubating the cell culture with 5 μM quinacrine in Hanks solution containing 10 mM HEPES at 37°C for 15 min. Quinacrine, a derivative of acridine, is a weak base that binds ATP with high affinity [23, 24]. After the incubation the cells were washed 5 times with Hanks solution and used to visualize vesicles using TIRF microscopy.

TIRF microscopy. To visualize and investigate the dynamics of the release from astrocytes of ATP-containing vesicles stained with quinacrine, TIRF microscopy was used. An inverted TIRF microscope (IX71, Olympus, Japan) equipped with an immersion oil lens with a high numerical aperture (60×/1.65 NA) and a cooled high-resolution camera (Hamamatsu, Japan) was used for this. Series of images were obtained and analyzed using the Olympus Cell software package tool (Olympus). The quinacrine fluorescence was excited using an argon laser at a wavelength of 488 nm, emission was recorded at a wavelength range 500−530 nm. To assess changes in the fluorescence intensity, an area of interest (ROI) was selected, in which fluorescent granules containing ATP were

Table 1. Genes and their primers

Gapdh	Forward 5'-ccacggcaagttcaacggcac-3'
	Reverse 5'-gatgatgacccttttggccccacc-3'
Ikk2	Forward 5'-gtctgggaggtgcccgagc-3'
	Reverse 5'-ggtccctgtggattatgtcagccgag-3'
Il1b	Forward 5'-gaagcagctatggcaactgtccctga-3'
	Reverse 5'-caggtcgtcatcatcccacgagtcac-3'
Tnfa	Forward 5'-ccgagatgtggaactggcagaggag-3'
	Reverse 5'-ttggccaggagggcgttgg-3'
Stat3	Forward 5'-cagctggacacgcgctacctg-3'
	Reverse 5'-ctgcttctccgtcactacggcag-3'
Socs3	Forward 5'-cgtgcgccatggtcaccca-3'
	Reverse 5'-gctgcccccctcgcact-3'
p53	Forward 5'-gcagagttgttagaaggcccagagg-3'
	Reverse 5'-gccgtcaccatcagagcaacg-3
Fas	Forward 5'-gtttggagttgaagaggagcgttcgt-3'
	Reverse 5'-cattggcacactttcaggacttggg-3'
Bcl2	Forward 5'-tggagatgaagactccgcgcccctga-3'
	Reverse 5'-cgtggcaaagcgtcccctcgcggt-3
Il6	Forward 5'-tgggactgatgttgttgacagccactg-3'
	Reverse 5'-ccaggtagaaacggaactccagaagacc-3'

detected. A decrease in the fluorescence intensity in the region of interest testified to the secretion of vesicles into the extracellular space. Experiments were performed at 37°C.

Isolation of total RNA. Total RNA was isolated from the primary culture of neurons using the Mag Jet RNA reagent kit (Thermo Scientific, USA) according to the manufacturer's instructions. The quality of the RNA was assessed by electrophoresis in 2% agarose gel in TBE buffer in the presence of ethidium bromide (1 µg/mL). The RNA concentration was measured using a NanoDrop 1000c spectrophotometer (Thermo Scientific, USA).

cDNA was synthesized using the RevertAid H Minus First Strand kit according to the protocol recommended by the manufacturer (Thermo Scientific). Single-stranded cDNA preparations were used as a template for real-time PCR analysis.

Real-time polymerase chain reaction (Real-time qPCR). RT-qPCR was performed in 25 µL of a mixture containing 5 µL of qPCRmix-HS SYBR (Evrogen, Russia), 1 µL (0.2 µmol) of each primer, 17 µL of water, and 1 µL of cDNA. Amplification was performed in a 48-well Dtlite5 amplifier (DNA Technology, Russia). At the beginning, denaturation was carried out for 5 min at 95°C, then 40 amplification cycles: denaturation at 95°C, 30 s; annealing at a primer-specific temperature (60−62°C), 20 s; elongation at 72°C, 30 s. Reporter fluorescence was read at the stage of elongation at a temperature of 62°C.

The RT-PCR data were analyzed using Dtlite software (DNA-Technologiya, Russia). Three independent RT-PCR experiments were performed (three independent hippocampal cell cultures). In all experiments, each cDNA sample was amplified in triplicate and average values were found. Primers specific for the investigated genes were selected using FAST PCR 5.4 programs and the NCBI Primer-BLAST system and were synthesized by Evrogen (Russia) (Table 1).

Gene expression was normalized to the control gene *GAPDH* encoding glyceraldehyde-3-phosphate dehydrogenase. The results were calculated in accordance with the standard method [25].

RESULTS

Agonists of α_2-adrenergic receptors inhibit OGD-induced global increase of $[Ca^{2+}]_i$ in astrocytes and protect cells from damage. We have previously shown that activators of α_2 adrenoreceptors, guanfacine and UK-14,304, depending on the exposure time (15 to 40 min), can inhibit the global $[Ca^{2+}]_i$ OGD-induced increase in hippocampal cells in vitro [11]. OGD causes Ca^{2+} signals similar in shape and amplitude in hippocampal neurons (Fig. 1a) and astrocytes (Fig. 1b), having a transient first phase and a global $[Ca^{2+}]_i$ increase at a second phase. The global $[Ca^{2+}]_i$ increase occurs in neurons (65 ± 25% of the cells in the field of view of the microscope) and astrocytes (57 ± 32%), is not synchronous, and can vary from culture to culture. Incubation of cell cultures with 3 µM guanfacine (GF) for 24 h significantly suppresses the amplitude of the global $[Ca^{2+}]_i$ increase in 90 ± 8% neurons (Fig. 1c). In 10 ± 2% neurons (Fig. 1c), the effect of guanfacine is less pronounced, and the global $[Ca^{2+}]_i$ increase is recorded in them, which begins 37−40 min after the onset of OGD, i.e., there is a 20-min lag period. In astrocytes, 24-h incubation with guanfacine causes inhibition of the primary OGD-induced Ca^{2+} signal by 65 ± 14% and a complete suppression of the global $[Ca^{2+}]_i$ increase (Fig. 1d) in all cells.

When cells were stained with propidium iodide prior the experiments (Fig. 1e, panel PI), it can be seen that the non-selective membrane permeability, as a sign in necrotic death, is recorded in single cells, the number of dead cells did not exceed 11 ± 5%, whereas during OGD there is a massive death of hippocampal cells in the network (Fig. 1e, panel PI + OGD). After OGD, of the total number of damaged cells (comprising more than 80% in the field of view of the microscope), 65 ± 23% of neurons and 82 ± 11% of astrocytes die on average (Fig. 1f, *black columns*). Incubation of cell culture for 24 h with 3 µM guanfacine reduces the number of hippocampal cells dying during OGD (Fig. 1e, GF line, PI + OGD), while the number of dying neurons decreases to 27 ± 12%, and astrocytes, to 11 ± 6% (Fig. 1f, *gray columns*).

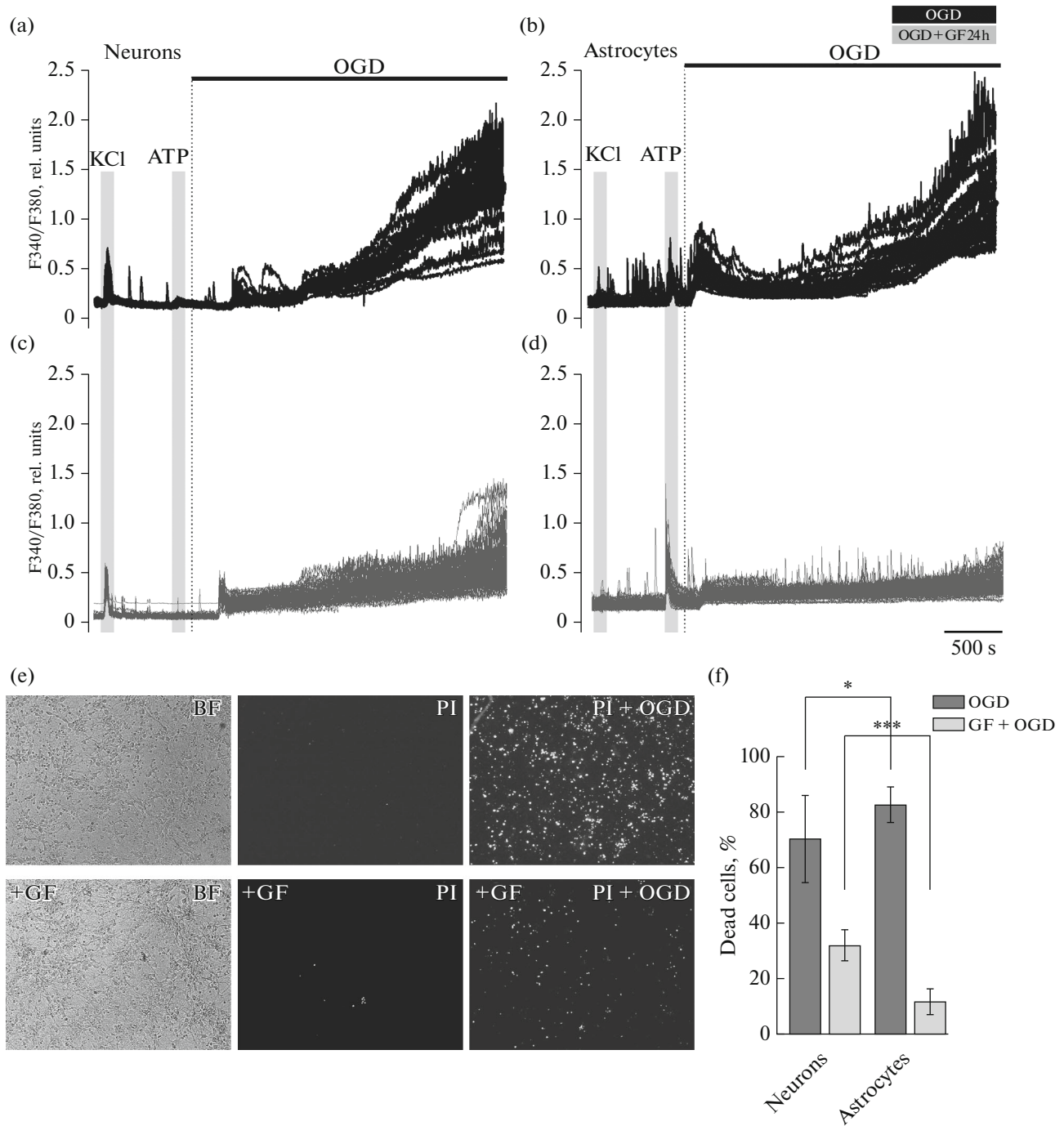Thus, a global $[Ca^{2+}]_i$ increase in the brain cells leads to their rapid death due to necrosis [26], and

Fig. 1. Neuroprotective effect of a 24-h incubation of hippocampal cells with 3 μM α_2-adrenergic receptor agonist guanfacine (GF). Ca^{2+} signals of neurons and astrocytes during 40-min OGD in control (a, b) and after 24-h incubation with 3 μM α_2-adrenergic receptor agonist guanfacine (c, d). Ca^{2+} signals of cells in single experiments obtained using the same cell culture are presented. (e) Images of hippocampal cell culture in transmitted light (BF) and propidium iodide fluorescence recording channel (PI) before the start of the experiment and 40 min after OGD (PI+OGD). The white dots are PI-stained nuclei of necrotic cells. (f) The average number of PI-stained cells that died due to the OGD-induced necrosis in neurons and astrocytes of the hippocampus in the absence of guanfacine (OGD) and after 24-h incubation with 3 μM guanfacine (GF+OGD) (mean % ± SE). *Differences are statistically significant, $p = 0.009$; ***differences are statistically significant, $p \leq 0.001$. Short-term applications of 35 mM KCl and 10 μM ATP were used to detect neurons and astrocytes, respectively.

inhibition of the increase in Ca^{2+} concentration by guanfacine correlates with a decrease in the percentage of dead cells, primarily astrocytes. These data indicate the existence of the Ca^{2+}-dependent mechanism of the neuroprotective action of guanfacine on astrocytes subjected to OGD.

Agonists of α_2-adrenergic receptors exert an anti-apoptotic effect during OGD and reoxygenation. In addition to the lack of oxygen and the absence of glucose, a second damaging factor in OGD is reoxygenation—the restoration of the normal partial pressure of oxygen in the medium. Usually, reoxygenation is accompanied by symptoms of hyperexcitation in neurons [27, 28], selective death of individual populations of GABAergic neurons [26], oxidative stress, necrosis and apoptosis [4, 29]. To investigate the anti-apoptotic action of guanfacine under OGD and acute reoxygenation (2 h after OGD), experiments were performed, the results of which are shown in Fig. 2. Cell cultures were preliminarily loaded for 1 h with a fluorogenic substrate of caspase-3, NucView-488. Prior OGD, NucView-488 fluorescence (Fig. 2a, column 0'), which indicates the apoptotic processes associated with the activity of caspase-3, was observed only in single cells. Then the cell culture was subjected to 40-min OGD (Fig. 2, designated OGD 40'), followed by 2-h reoxygenation (designated 2 h. Reox.) performed by replacing the OGD medium with the medium saturated with oxygen. During the whole experiment, NucView-488 fluorescence was recorded with a frequency of 1 frame in 30 s (Fig. 2b). In a control experiment, in which the cells were not exposed to OGD and reoxygenation, a slight appearance of NucView-488 fluorescence (Fig. 2b, curve *1*) was observed after 1—1.5 h of the fluorescence recordings, which may be due to the photodamaging effect of the light used for fluorescence excitation. During OGD, the fluorescence of NucView 488 in cells due to activation of caspase-3 was noticeable already in 10—20 min, and an exponential increase in intensity was observed over the next 40 min (Fig. 2b, curve *2*). During reoxygenation, the caspase-3 activity increased 4—5-fold, as compared with the level recorded during OGD. At the same

time, the number of cells, in which NucView-488 fluorescence appeared, reached 80—95% of the total number of cells in the field of view of the microscope and was subsequently taken as 100% (Fig. 2c). Cell cultures incubated for 24 h with micromolar concentrations of guanfacine were characterized by a weaker activation of caspase-3 during OGD and reoxygenation (Fig. 2a), as is also indicated by the averaged time course of the fluorescence intensity (Fig. 2b, curve *3*), the values of which did not exceed the control (Fig. 2b, curve *1*). During OGD, caspase-3 was activated in $20 \pm 4\%$ of cells incubated with guanfacine, and during reoxygenation, the number of fluorescent cells increased up to $22 \pm 3\%$ (Fig. 2c), in stark contrast to 80—95% in experiments without guanfacine. The anti-apoptotic effect was partially reversed in the presence of 1 μM of Bafilomycin A1, an inhibitor of V-ATPase, added during OGD (Fig. 2a, BafA1 + GF). Against BafA1, caspase-3 activation occurred predominantly during reoxygenation (Fig. 2b, curve *4*), although the fluorescence level of NucView-488 was 50% higher than the control, but the number of cells, in which apoptosis was activated, reached 40—43% during the reoxygenation period (Fig. 2c). Consequently, BafA1 abolished the protective effect of guanfacine by increasing the percentage of cells with induced apoptosis that develops as a result of reoxygenation.

The anti-apoptotic effect of guanfacine during OGD and reoxygenation correlates with a change in the basic level of the gene expression of apoptosis regulators (Fig. 2d). After 24-h incubation of cell cultures with 3 μM guanfacine, the expression of anti-apoptotic genes *Stat3* and *Bcl-2* is increased by 120% and 19%, respectively, while the level of pro-apoptotic genes is significantly reduced: *Socs-3*, by 50%; *p53*, by 96%, and *fas*, by 72%. In addition, after 24-h incubation of cells with 3 μM guanfacine, IKK expression (IκB kinase, Fig. 2d), which is involved in the regulation of apoptosis and inflammation, decreases by 58% [30]. The IKK inhibitor (BMS 345541, 5 μM) added 24 h before the experiments produced a neuroprotective effect similar to that of guanfacine (Fig. 2e), i.e., a decrease in the number of dead cells in OGD. At the

Fig. 2. Anti-apoptotic effect of 24-h incubation of hippocampal cells with α_2-adrenergic receptor agonist guanfacine. The role of the pool of acidic secretory vesicles and IKK. (a) Images of hippocampal cell cultures loaded with fluorogenic caspase-3 substrate NucView-488, and incubated for 24 h with 3 μM guanfacine (+GF) and in the presence of 1 μM bafilomycin A1 (BafA1) and guanfacine (BafA1+GF). Bafilomycin A1 was added to the cells during OGD and reoxygenation. Images are presented before the start of the experiments (0'), 40 min after OGD (40' OGD), and 2 h after reoxygenation (2 h Reox.). Numbers 1—4 correspond to the curves presented in panel (b). (b) Hydrolysis of the fluorogenic substrate of caspase-3 (NucView-488) during OGD (40 min) and reoxygenation (Reox., 2 h), indicating the induction of apoptosis in hippocampal cells. Designations: *1,* control (cell culture was not exposed to OGD and reoxygenation); *2,* cells were subjected to OGD (40 min) and reoxygenation (2 h); *3,* cells were incubated for 24 h with 3 μM guanfacine after OGD and reoxygenation; *4,* cells were incubated for 24 h with 3 μM of guanfacine, 1 μM of bafilomycin A1 was added during OGD and reoxygenation. (c) The number of cells (%) with NucView-488 fluorescence. The number of cells with apoptosis in the group without the addition of guanfacine is taken as 100%. (d) Effect of 24-h incubation of hippocampal cells with 3 μM guanfacine on the expression of genes regulating apoptosis. (e) Images of hippocampal cell cultures incubated for 24 h with an IKK inhibitor (BMS 345541, 5 μM): BF, in transmitted light; PI, fluorescence channel before experiments; PI+OGD, fluorescence channel 40 min after OGD. (f, g) Ca^{2+} responses of neurons (f) and astrocytes (g) of the hippocampus to OGD (40 min) after 24-h incubation with 5 μM of IKK inhibitor BMS 345541. Ca^{2+} signals in single experiments, in the same cell culture are presented. Short-term applications of 35 mM KCl and 10 μM ATP were used to detect neurons and astrocytes, respectively.
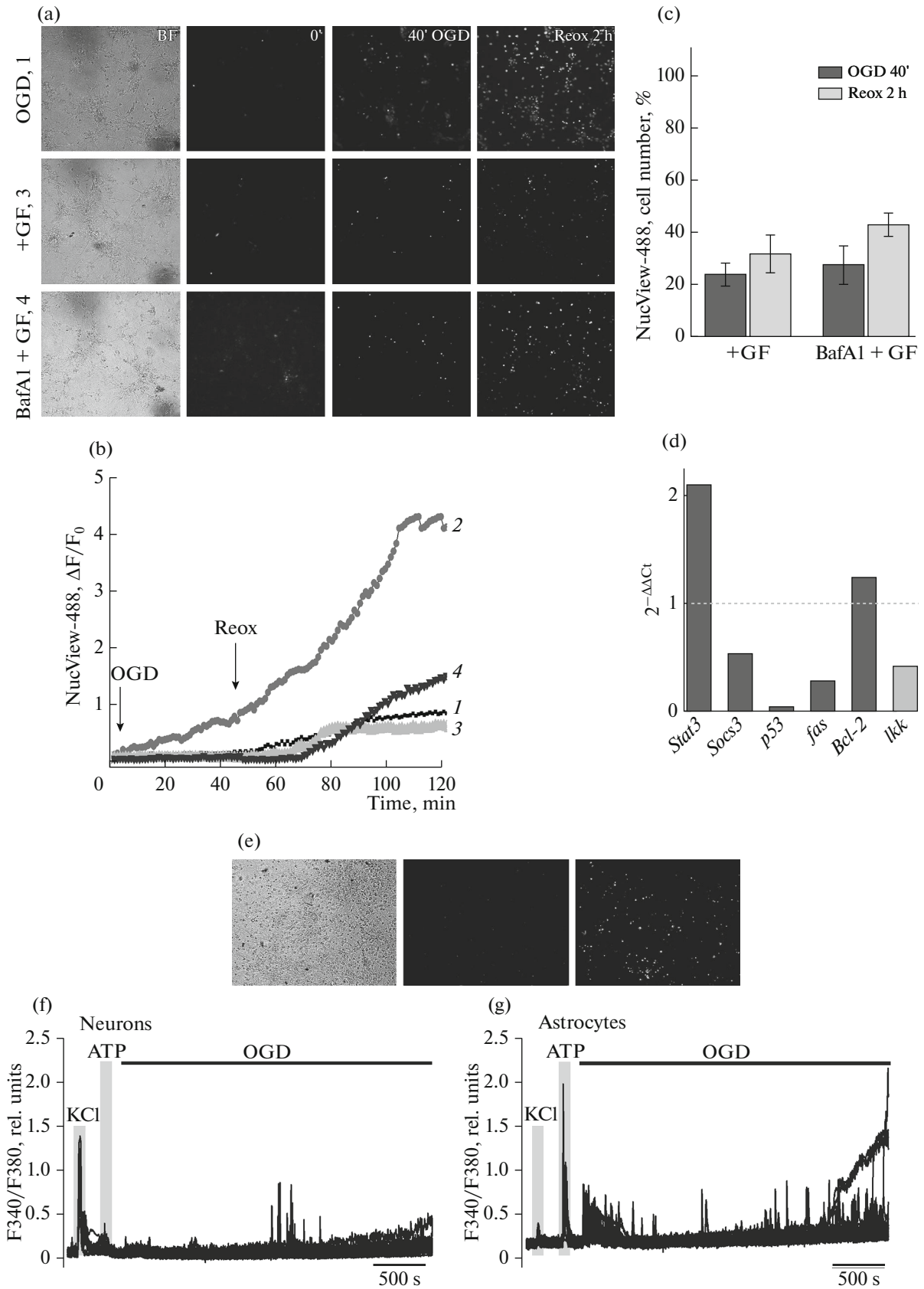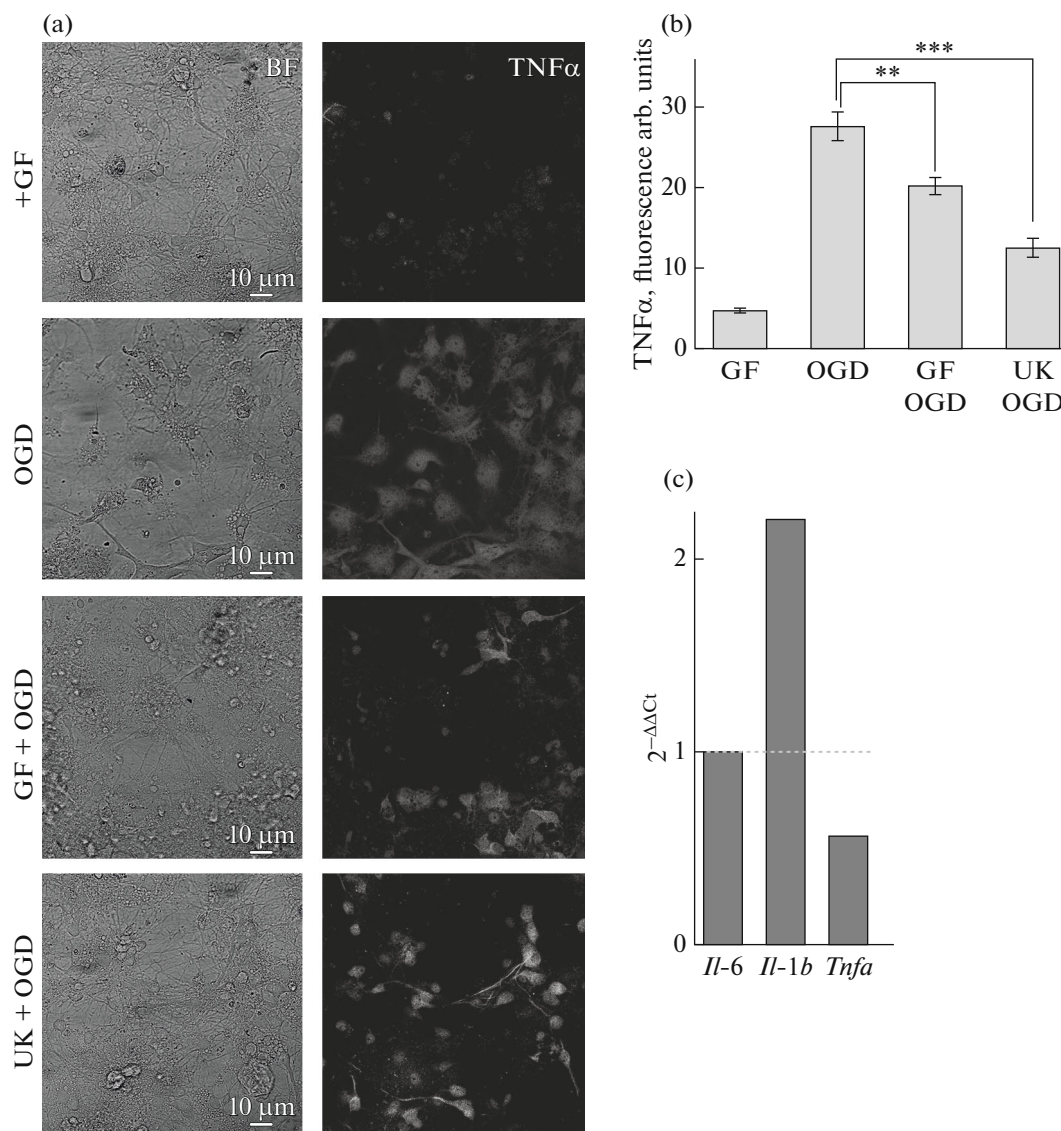
Fig. 3. Anti-inflammatory effect of 24-h incubation of cells with α_2-adrenergic receptor agonists. (a) Staining of cell cultures with antibodies against TNFα. (b) Level of fluorescence (in arbitrary units) of secondary antibodies to primary antibodies against TNFα in cells after incubation with α_2-adrenergic receptor agonists. (c) Effect of 24-h incubation of hippocampal cells with 3 μM guanfacine on the expression of pro-inflammatory genes. Designations: BF, images of cell cultures in transmitted light; TNFα, fluorescence of secondary antibodies to primary antibodies against TNFα; +GF, cells were incubated for 24 h with 3 μM guanfacine; OGD, cells after 40-min OGD and 24 h of reoxygenation; GF+OGD and UK+OGD, images of cultures after 40-min OGD and 24 h reperfusion, incubated for 24 h in the presence of 3 μM guanfacine and 1 μM UK 14,304, respectively. **Differences are statistically significant, $p = 0.005$; ***differences are statistically significant, $p \leq 0.001$.

same time, neurons show a complete suppression of OGD-induced $[Ca^{2+}]_i$ increase (Fig. 2f). In most astrocytes, BMS 345541, like guanfacine, does not affect the generation of OGD-induced Ca^{2+} signal during the first phase, but inhibits the global increase of $[Ca^{2+}]_i$ in the overwhelming number of cells (Fig. 2g).

Thus, the activator of α_2-adrenergic receptor guanfacine has an anti-apoptotic effect on the hippocampal cells during OGD and reoxygenation: it inhibits

caspase-3 and regulates the expression of anti- and pro-apoptotic genes.

Agonists of α_2-adrenergic receptor inhibit OGD-induced accumulation of pro-inflammatory cytokines in neuroglial culture. Activation of apoptosis in the brain during ischemia−reoxygenation is closely associated with pro-inflammatory signaling cascades [31]. Hippocampal cells incubated in the presence of 3 μM guanfacine for 24 h (Fig. 3a, + GF) and then stained with antibodies against TNFα (Fig. 3a) are character-

ized by the presence of secondary antibody fluorescence only in single cells. After 40-min OGD the cells were returned to the CO_2 incubator for 24 h and then stained with antibodies against TNFα (Fig. 3a, OGD), the fluorescence was recorded in 80% of the cells in the field of view, indicating the development of a cellular inflammatory response. However, cell cultures that were pre-incubated for 24 h with α_2-adrenergic receptor agonists, guanfacine (3 μM) or UK-14,304 (1 μM), are characterized by a smaller number of cells stained with antibodies against TNFα Fig. 3a, GF+OGD, UK+OGD), which indicates the suppression of inflammation by these agonists. The level of fluorescence of secondary antibodies reflects the amount of intracellular protein. After 24-h incubation of cells with α_2-adrenergic receptor agonists, there is a decrease in the amount of TNFα protein compared to OGD without agonists (Fig. 3b).

PCR data show that incubation of cells with 3 μM guanfacine for 24 h leads to a decrease in the expression of the gene encoding TNFα by 46% (Fig. 3c), which is in good agreement with the immunocytochemical staining (Figs. 3a, 3b). The level of pro-inflammatory cytokine IL-6 does not change after incubation with guanfacine, and the expression of the gene encoding the pro-inflammatory cytokine IL-1b increases by 130% (Fig. 3c) and can have a preconditioning effect on neuroglial cultures.

Thus, α_2-adrenergic receptor agonists, guanfacine and UK-14,304, have a neuroprotective effect in OGD and reoxygenation by suppressing the inflammatory process and inhibiting the expression of the gene and TNFα protein.

α_2-Adrenergic receptor agonists induce ATP secretion by astrocytes and suppress hyperexcitation of neuronal networks. Adrenergic receptors are widely distributed in the brain, but the expression of α_2-adrenergic receptors in astrocytes is 2−3 times greater than in neurons [32]. Application of α_2-adrenergic receptor agonists, guanfacine (3 μM, GF) and UK-14,304 (1 μM, UK) to the rat hippocampal cultures caused Ca^{2+} responses in astrocytes (Fig. 4a, *black curves*) but not in neurons (Fig. 4a, *gray curve*). Astrocytic Ca^{2+} signals in most cases had the appearance of a pulsed increase in $[Ca^{2+}]_i$ or were irregular oscillations. The basal level of $[Ca^{2+}]_i$ after application and washing of α_2-adrenergic receptor agonists in the vast majority of experiments was maintained at a slightly elevated level. One of the main ionotropic excitatory receptors involved in the damaging effect of OGD on neuronal networks are NMDA receptors. An increase in the concentration of Ca^{2+} ions in the cytosol of neurons and astrocytes during ischemia causes the release of glutamate by neurons and gliotransmitters, primarily ATP [33], by astrocytes.

The removal of magnesium ions from the medium (Mg^{2+}-free) causes the appearance of synchronous Ca^{2+} oscillations of high frequency in cultured hippocampal neurons, a sign of hyperexcitation. The application of 5 μM ATP suppressed these oscillations and caused a transient Ca^{2+}-response in astrocytes (Fig. 4b, *gray curve*). Using TIRF microscopy and staining of ATP-containing vesicles in astrocytes with the vital quinacrine probe (Fig. 4c), we were able to show that the application of the α_2-adrenergic receptor agonist dexmedetomidine at a concentration of 1−10 μM (Fig. 4c, +Dex) causes secretion of ATP registered as a rapid and significant decrease in quinacrine fluorescence intensity in the astrocyte soma (Fig. 4d).

The application of 3 μM guanfacine with Mg^{2+}-free medium causes Ca^{2+} signal in astrocytes and leads to the rapid suppression of Mg^{2+}-free induced Ca^{2+} oscillations in neurons (Fig. 4e, *gray curve*). Thus, selective α_2-adrenergic receptor agonists induce transient Ca^{2+} signal in hippocampal astrocytes and Ca^{2+}-dependent ATP secretion, which leads to suppression of the Mg^{2+}-free induced hyperexcitation of neuronal network. Apirase (35 U/mL), an enzyme that hydrolyzes ATP, applied before the Ca^{2+} oscillations induced in neurons by Mg^{2+}-free solution, does not affect the generation of oscillations but reduces their frequency (Fig. 4e). At the same time, the application of 3 μM of guanfacine with apyrase still causes the generation of Ca^{2+} signal in astrocytes but does not lead to the suppression of Ca^{2+} oscillations, which indicates the involvement of guanfacine-dependent ATP secretion in suppressing hyperactivity in neurons.

Bafilomycin A1 (BafA1, 1 μM) that inhibits V-ATPase in acidic compartments, including ATP-containing vesicles [34], partially reverses the effect of guanfacine (Fig. 4f) by inhibiting Ca^{2+} oscillations in neurons in 3−5 min, whereas with the first application of guanfacine, the suppression of oscillations occurs simultaneously with the generation of Ca^{2+} signals by astrocytes. An increase in incubation time with Baf A1 up to 1 h removed the inhibitory effect of guanfacine (Fig. 4g), confirming the hypothesis about the leading role of ATP-containing vesicles in astrocytes in the inhibition of hyperexcitation of neuronal networks by α_2-adrenergic receptor agonists.

Thus, investigation of the neuroprotective properties of α_2-adrenergic receptor agonists have shown their anti-inflammatory and anti-apoptotic action during ischemia and reoxygenation, which is realized by changing the base expression of the regulator genes of these processes and the post-ischemic level of proteins encoded by them. At the level of Ca^{2+} signaling, the effect of α_2-adrenergic receptor agonists leads to inhibition of the global increase in $[Ca^{2+}]_i$ in the hippocampal cells during OGD in vitro and to a decrease number of cells that die by necrosis. Ca^{2+}-dependent ATP secretion by astrocytes is involved in the mecha-
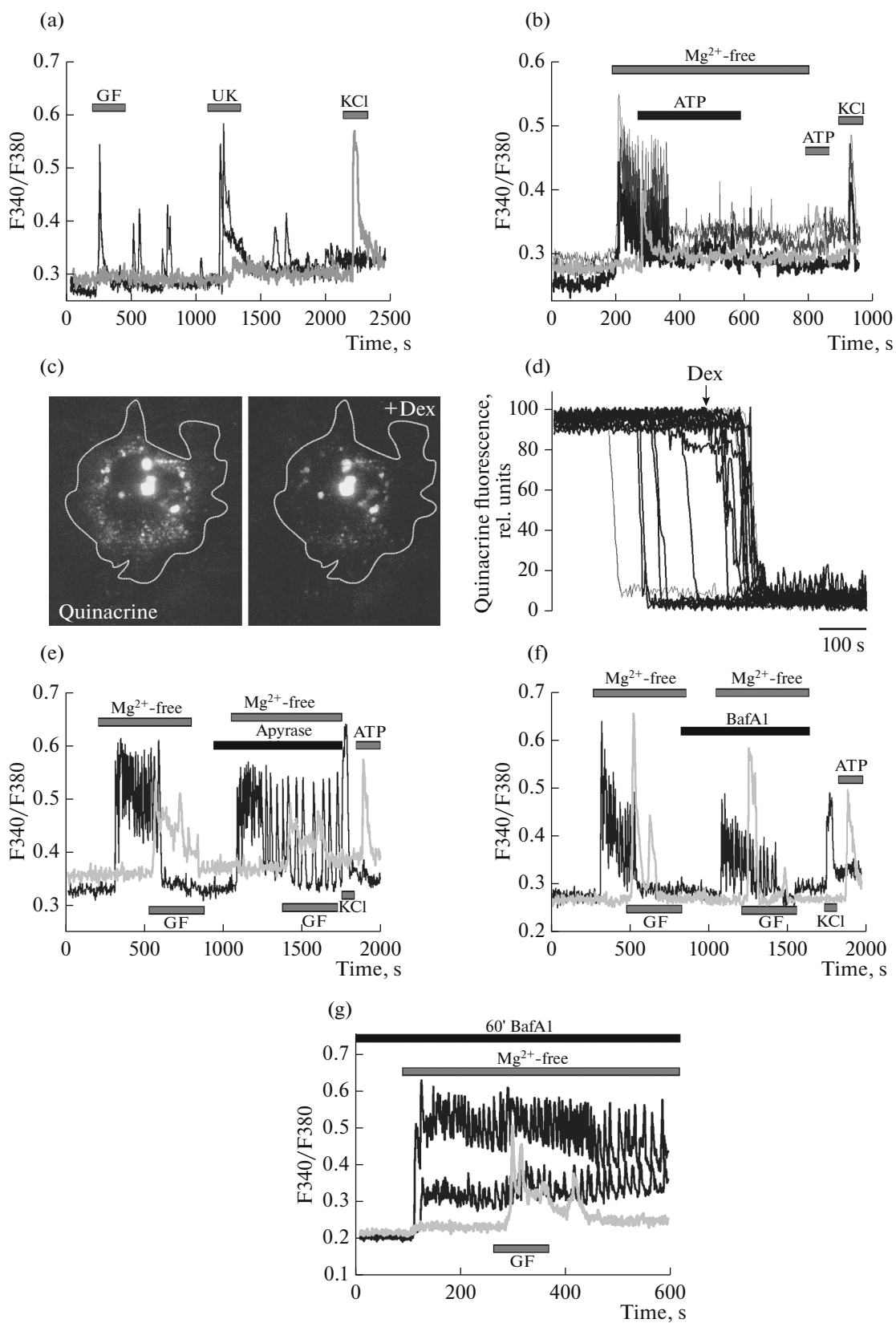
(a)

(b)

(c) Quinacrine +Dex

(d) Dex

(e)

(f)

(g)

Fig. 4. The mechanism of the neuroprotective action of α_2-adrenergic receptor agonists during epileptiform activity. (a) Application of selective α_2-adrenergic receptor agonists guanfacine (3 µM GF) and UK-14,304 (1 µM, UK) to hippocampal cell cultures. *Light gray curve* is an averaged Ca^{2+} signal of several dozen neurons. *Black curve* is an averaged Ca^{2+} signal of several dozens of astrocytes. (b) Application of 5 µM ATP during epileptiform activity of the hippocampal neurons (*black* and *dark gray curves*), caused by Mg^{2+}-free medium, leads to the suppression of the hyperexcitation of these neuronal network and an increase in the Ca^{2+} concentration in the astrocytic cytosol (*dark gray curve*). (c) Images of near-membrane localization of quinacrine-stained ATP-containing vesicles obtained by TIRF-microscopy before and after the application of 10 µM dexmedetomidine (+Dex). A single astrocyte is presented. (d) Time course of secretion of ATP-containing vesicles obtained using TIRF-microscopy, reflecting an increased secretion (decrease in quinacrine fluorescence) upon application of 10 µM dexmedetomidine (Dex). Secretion of single ATP-containing vesicles prior to exposure of dexmedetomidine is a normal spontaneous secretory activity of astrocytes. (e) The application of guanfacine (3 µM, GF) leads to the generation of Ca^{2+} signals in astrocytes and the suppression of epileptiform Ca^{2+} oscillations in neurons in a Mg^{2+}-free medium. Guanfacine-produced suppression of hyperexcitation of neurons is canceled by apyrase (apyrase, 35 U/mL) hydrolyzing ATP. (f) Guanfacine-produced suppression of neuronal hyperexcitation in a Mg^{2+}-free medium is partially canceled in the presence of a V-ATPase inhibitor bafilomycin A1 (BafA1, 1 µM). (g) Ca^{2+} oscillations of neurons in Mg^{2+}-free medium are not suppressed by the application of 3 µM guanfacine (GF) after a 60-min pre-incubation of the cell culture with 1 µM bafilomycin A1. Short-term applications of 10 µM ATP and 35 mM KCl at the end of the experiments were performed to detect astrocytes and neurons in culture, respectively. Ca^{2+} signals of single cells characteristic of most neurons and astrocytes in the network are presented.

nism of the protective action of α_2-adrenergic receptor agonists.

DISCUSSION

This work shows the complex protective effect of α_2-adrenergic receptor agonists on the survival of neurons and astrocytes of the hippocampus under conditions of oxygen-glucose deprivation in vitro.

One of the effects of α_2-adrenergic receptor activation in the mixed hippocampal neuroglial culture is the secretion of ATP by astrocytes. This process is the fundamental mechanism of communication of glial cells among themselves and with other types of cells in the brain [35]. It is known that in ATP vesicles it can be packaged simultaneously with other neurotransmitters, such as acetylcholine, adrenaline, etc. [36], which can indicate the secretion of several active substances at once upon activation of α_2 adrenoreceptors. Astrocytes are known to be able to secrete glutamate in the form of vesicles via the Ca^{2+}-dependent mechanism [37]; however, glutamate is stored in vesicles other than ATP-containing ones [38]. When guanfacine is applied, there is no increase in Ca^{2+} oscillations in neurons, as well as excitotoxic effects, on the contrary, there is a complete suppression of hyperexcitation, which indicates the secretion of the pool of ATP-containing vesicles upon activation of α_2 adrenoreceptors. This assumption is confirmed by the results of TIRF microscopy.

ATP has a rather diverse effect on neuroglial networks [35], but in the context of this work, the suppression of neuronal activity shown by other authors [39] is most significant and interesting. It is established that ATP secreted by astrocytes suppresses the activity of neural networks. A similar effect was also shown by us in experiments where the suppression of calcium oscillations by guanfacine in neurons induced by the application of Mg^{2+}-free medium was detected. This effect was canceled in the presence of a vesicular ATPase inhibitor, bafilomycin A1, or an ATP-degrad-

ing enzyme apyrase, indicating that vesicular transmission is involved in this process. A similar phenomenon was described earlier in [40]. Activation of adenosine receptors by adenosine, resulting from the extracellular degradation of ATP, can be considered as one of the putative mechanisms of suppression of neuronal activity by ATP secreted from astrocytes [33]. ATP secreted by astrocytes can also affect glial cells by activating P2Y1 and P2X7 receptors. Activation of these receptors leads to an increase in the concentration of cytosolic calcium due to mobilization from internal stores (P2Y1-mediated action) or influx from the outside (via P2Y7 receptors) and further calcium wave propagation with the subsequent release of gliotransmitters. The physiological role of these phenomena still remains a subject of discussion [33], which makes it difficult to put forward hypotheses about the possible contribution of astrocytic ATP secretion to the protective effect of α_2 agonists in ischemia.

A possibility of the involvement of α_2 adrenoreceptors in the glutamate secretion regulation should also be considered. The preferential presynaptic localization [41], as well as participation in the regulation of the activity of potassium and voltage-gated calcium channels [42], allows α_2-adrenergic receptors to change the intensity of neurotransmitter secretion [43], excitatory in particular [44]. This fact may be one of the possible explanations for the suppression of Ca^{2+} signals at OGD after 24-h incubation with α_2-adrenoreceptor agonists, as well as the suppression of high-frequency oscillations in neurons induced by an Mg^{2+}-free medium. This assumption is supported by the results of a number of investigations, in which α_2-adrenoreceptor agonists inhibit the secretion of excitatory amino acids, such as glutamate and aspartate [45, 46].

At the same time, the effects of prolonged incubation are difficult to explain by secreting ATP, lactate (or reduced glutamate release), since the release of transmitters from both neurons and glial cells is a quantized process [47]. In addition, desensitization

processes should be taken into account aimed at reducing over-activation of receptors [48], as well as the limited pool of neurotransmitters, which can be depleted quickly enough, even despite recycling through reuptake mechanisms [49]. Therefore, the conclusion is that the above effects of guanfacine and other α_2-adrenergic receptor agonists, coupled with the regulation of vesicular secretion of gliotransmitters, can be viewed as rapid effects (minutes). The presence of rapid effects can explain the therapeutic potential of using α_2 agonists for diseases associated with hyperexcitation of neuronal networks, for example, for epilepsy [50], as well as for using these compounds as anesthetics [18]. However, one should take into account the long-term effects, the development of which can be observed during prolonged incubation and occur 1 h after the application of agonists. It is logical to assume that prolonged incubation with α_2-adrenoreceptor agonists leads to the activation of signaling cascades involved in the regulation of transcription of the genes regulating apoptosis and the inflammatory response of the cells. Activation of these cascades can make a significant contribution to the implementation of the neuroprotective effect of α_2 agonists.

It is known that cAMP regulates the activity of calcium channels, the conductivity of which decreases with decreasing cAMP concentration [51], thus weakening the total influx of calcium ions. The activity of calcium-activated potassium channels of low conductivity, on the contrary, increases with decreasing concentration of cAMP, increasing the outflow of potassium ions [52]. Thus, reducing the concentration of cAMP in the cytosol of neurons contributes to hyperpolarization and the prevention of calcium overload. In addition, through the phosphorylation of the GluA1 subunit of the AMPA receptor by the Ser845 residue, protein kinase A regulates the subunit traffic and their incorporation into the membrane [53]. Reduced activity of protein kinase A, due to the low level of cAMP in the cytosol (and, as a result, reduced intensity of CREB phosphorylation), can lead to the fact that the number of functionally active AMPA receptors on the membrane can decrease [54]. Since the primary depolarization required for the removal of the magnesium block of NMDA receptors is also due to the influx of cations through AMPA receptors [55], the contribution of NMDA receptors to the total depolarization and the influx of calcium ions from the external environment can also be reduced. Summing up these facts, we can assume that the reduced amplitude and rate of global increase in $[Ca^{2+}]_i$ during the second phase of the Ca^{2+} response of neurons to OGD can be explained by a decrease in the concentration of cAMP in the cytosol of cells caused by activation of α_2 adrenoreceptors.

In our experiments, we showed that the number of dead cells after simulating ischemia—reperfusion in cultures preincubated for 24 h with guanfacine was sig-

nificantly less than in cultures without guanfacine. On the basis of these data we conclude that α_2-adrenoreceptor agonists have a powerful anti-apoptotic effect. A possible explanation for such a high cell survival is the activation of NF-kB. This transcription factor is located at the "intersection" of many intracellular signaling cascades, among which the following should be noted: cascades involving cAMP, TNFα, IL-1, signaling pathway PI3K-Akt [56−58]. As for the cAMP-linked pathway of NF-kB activation, as a result of inhibition of the adenylate cyclase by the $G_{i\alpha}$ subunit of α_2 adrenoreceptors, the number of cAMP and the activity of protein kinase A should be significantly reduced, which eliminates this signaling cascade.

It should be noted that α_2-adrenergic receptor agonists can alter the balance of expression of pro- and anti-inflammatory cytokines [59]. The amount of IL-1β mRNA increased significantly after 24-h incubation with guanfacine, while no change in the amount of IL-6 mRNA was detected in our experiments. A significant increase in expression could lead to increased secretion of IL-1, followed by activation of the corresponding receptors (IL-1R). The signaling cascade of this receptor is associated with the activation of JAK and STAT [60]. We have shown that the expression of Stat3 increased after incubation of cultures with guanfacine, which may indicate a significant contribution of this pathway.

The decrease in TNFα expression (including the decrease in protein levels), shown in our experiments, is consistent with the data obtained on immune cells [59]. Such a decrease may be associated with the activation of astrocytes. Thus, it has been established that activation of astrocytes plays a protective role during neurodegenerative processes resulting from inflammatory processes in the hippocampus during ischemia, reducing the level of TNFα [61]. Common to the TNFα and PI3K-Akt signaling pathways is that the activation of NF-kB occurs via IKK [62], but the expression of IKK was reduced in our experiments. Therefore, most likely, the contribution of TNFα to the activation of the NF-kB upon activation of α_2 adrenoreceptors is minimal, whereas the PI3K-Akt cascade may be partially involved in this process. It is known that activation of PI3-kinase can also occur directly through the $G_{i-\beta\gamma}$ subunit of the α_2-adrenergic receptor [63]. We have previously shown that activation of PI3 kinase promotes the survival of neuroglial culture cells upon oxygen-glucose deprivation [11]. The participation of the PI3K-Akt cascade in the implementation of the anti-apoptotic action of α_2 adrenoreceptors is evidenced by the fact that the expression of genes *p53* and *fas* in our experiments was reduced, possibly due to the activation of this signaling cascade. Akt-induced reduction of the *p53* expression is known to contribute to the survival of hippocampal neurons during hypoxia [64]. It should be noted that the expression of anti-apoptotic factor Bcl-2 increased

after prolonged incubation with guanfacine. Bcl-2 may be known to play an important role in suppressing apoptosis in neuronal cells after excitotoxic damage or ischemia [65]. Activation of this factor can occur both within the PI3K-Akt [66], and JAK-STAT cascade (with the participation of STAT-3) [67]. On the basis of the foregoing it can be concluded that the anti-apoptotic effect of α_2-adrenoreceptor agonists can be due to both PI3K-Akt and JAK-STAT cascades and include activation of the factors NF-kB and Bcl-2; IL-1 in this case can be considered as a protective substance.

Much attention is paid to the effect of α_2-adreno-receptor agonists on neurons. However, considering such a complex system as neuroglial networks, one cannot but take into account glial cells. In this paper, we have shown the complex effect of α_2-adrenoreceptor agonists on both neurons and astrocytes. In addition to long-term effects associated with changes in gene expression and suppression of apoptosis and the inflammatory response, guanfacine and other α_2 agonists have been shown to activate a rapid protective effect consisting of the stimulation of vesicular ATP secretion by astrocytes, which suppress hyperexcitation in neurons.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

All procedures were performed in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC) and the Declaration on humane treatment of animals. The Protocol of experiments was approved by the Commission on Bioethics of Institute of Cell Biophysics, Russian Academy of Sciences.

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