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Effect of the Inhibitory Neurotransmitter Glycine on Slow Destructive Processes in Brain Cortex Slices under Anoxic Conditions

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Abstract—Slow destructive processes in brain cortex were studied under deep hypoxia (anoxia). Study of the character and dynamics of DNA destruction showed that apoptosis and necrosis run in parallel under the experimental conditions. These processes typically develop in tens of hours. A similar conclusion was reached from electron microscopic study of the tissue ultrastructure. More detailed study revealed that a relatively rare type of apoptosis not involving cytochrome *c* release from the intermembrane space of mitochondria and not associated with opening of the mitochondrial nonspecific pore occurs under the experimental conditions. As this is occurring, the process can be slowed by high concentrations of glycine, an inhibitory neurotransmitter. The study of DNA destruction demonstrated that high concentrations of glycine selectively slow apoptosis but have almost no effect on necrosis. Glycine also drastically decreases changes in the tissue ultrastructure, particularly of mitochondria, arising under anoxia. Glycine does not notably influence the mitochondrial oxidative phosphorylation system. Study of impairment of mitochondrial function demonstrated that the oxidative phosphorylation system is not disturbed for 1 h, which is several times longer than the inhibition time of brain function under deep hypoxia. The mitochondrial respiratory system is preserved for a relatively long time (24 h). Malate oxidase activity is deactivated after 48 h. The succinate oxidase fragment of the mitochondrial respiratory chain proved especially resistant; it retains activity under anoxia for more than 72 h. A possible mechanism of the effect of high glycine concentrations is discussed.

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Key words: brain cortex tissue, glycine, mitochondrion, apoptosis, necrosis, anoxia

Glutamate receptors are known to be hyperactivated in nervous tissue under hypoxia. Induction of apoptosis and degeneration of nerve tissue are found to be a consequence of hyperactivation of these receptors [1, 2]. Glycine at high concentrations is an inhibitory neurotransmitter inhibiting the hyperactivation of these receptors [3]. Possibly for this reason, glycine protects nerve tissue under hypoxic conditions. In particular, a glycine-stimulated increase in lifetime of cortical neurons was observed under hypoxic conditions [4]. It has been demonstrated by *in vivo* experiments that an elevation of resistance to hypoxia in mice arising under short-term decrease in oxygen level in atmospheric air is accompanied by the elevation of free glycine concentration in the brain [5]. Moreover, the effectiveness of glycine has been demonstrated in treatment of patients with ischemia accompanied by drastic decrease in partial oxygen pressure in the vicinity of the nidus of disease [6]. It was thus important to identify the brain cortex process whose development under anoxia is blocked or retarded under the influence of high concentration of glycine. Two tasks were carried out to solve this problem.

In the first stage, the character and dynamics of anoxic destruction of important biochemical systems was determined, e.g. genetic material decay and disturbance of mitochondrial energy processes; also, changes in ultrastructure of brain cortex under anoxia were characterized in brain slices. The characteristics of a model system

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enabling study of the effects of physiologically active substances on slow processes taking place in brain tissue under anoxia were obtained. Use of this model in the second stage of the work revealed a destructive process that is retarded by glycine under anoxia. Programmed cell death (apoptosis) were identified as such a process; it was registered by internucleosomal DNA fragmentation as well as by mitochondrial ultrastructure changes in the tissues.

MATERIALS AND METHODS

Conditions of tissue incubation. The experiments were done on wild type white rats 150-180 g in weight. "Incomplete decapitation" was carried out so that the cephalic artery was intact, through which perfusion of the brain was performed. The perfusion solution was as follows: 120 mM NaCl, 3 mM KCl, 10 mM Hepes-Tris, pH 7.4, the solution temperature was 37°C, the perfusion duration was 5 min. Then the brain was isolated and placed into the following incubation medium: 300 mM sucrose, 0.25 mM EDTA, 10 mM Hepes-Tris, pH 7.4 (M1). The cortex was separated, placed on a Petri dish with cold buffer (0°C) on ice, and cut into fragments of 2-3 mm^3. The fragments were put into tubes with preliminarily prepared anoxic incubation medium prepared by bubbling with nitrogen for 40 min. The tubes were sealed after placing the fragments into them. The incubation was carried out at 20°C; the incubation time varied from 1 h to several days. The pH of the medium was measured after the incubation. The pH value did not decrease below 7.1 during the experiments.

DNA extraction and electrophoresis. DNA was extracted by the method described in [7] with slight modifications. After the incubation under anoxic conditions, the brain slices were put in liquid nitrogen and pulverized with a pestle, then suspended in lysing buffer solution (50 mM Tris-HCl, pH 7.4, 25 mM EDTA, 1% sodium dodecyl sulfate) at room temperature. The lysates were deproteinized by shaking with phenol, and nucleic acids were precipitated with one volume of isopropanol from the water phase separated by centrifugation. After dissolving the pellet in TE-buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA), a solution of RNase A was added to the mixture to its final concentration of 100 μg/ml, and the mixture was incubated for 20 min at 37°C. DNA was precipitated with one volume of isopropanol after repeated deproteinization. DNA preparations were subjected to the electrophoresis in 1% agarose gel in 40 mM Tris-acetate buffer, pH 8.0, containing ethidium bromide (0.5 μg/ml). Photographs of the gels were obtained with a Shater analyzer of biopolymer mixtures (Deltatex, Russia).

Electron microscopy. For electron microscopic study, the rat brain slices after their incubation under anoxic conditions for 3 days in non-modified medium or in glycine-containing medium were fixed with 2.5% glutaraldehyde solution in the incubation medium at room temperature for 2 h. Slices of the same rat brain cortex area fixed immediately after the sacrifice of an animal and separation of its brain in the same way as the experimental material served as a control.

After washing with the medium, the experimental samples were completely fixed with 2% osmium tetroxide solution in the same medium for 2 h, gradually dehydrated with ethanol solutions in water of increasing concentrations, and then in absolute ethanol and acetone, and embedded in Epon 812 resin. Ultrathin sections were prepared on an Ultratom LKB-3 (Ultra Nova, Sweden), contrasted with water-saturated uranyl acetate and lead citrate, and examined under a Tesla BS-500 electron microscope (Czechoslovakia) under 90 kV accelerating power. The areas of interest were photographed on a film with magnification of 10,000 and 18,000. Structural information was given in digital form at resolution of 600 pixels per inch using an Epson 3200 scanner (Epson, Japan).

Homogenate preparation. Slices of brain cortex from one animal were taken from tubes and washed in homogenization medium cooled to 0°C; thereafter they were placed in a homogenizer with 3 ml of medium and homogenized for 4-5 passes. To study respiratory activity, the homogenates were made in the following medium: 210 mM mannitol, 70 mM sucrose, 10 mM Hepes-Tris, 1 mM EDTA, 0.1% BSA. To study spectral characteristics, homogenates were prepared in the following medium: 150 mM KCl, 10 mM Hepes-Tris, 1 mM EDTA (M2) or in the incubation medium (M1). Before the registration of spectra, the homogenates were centrifuged at 20,000*g* for 10 min, then the supernatant was removed, and the volume of homogenization medium equal to the volume of the removed supernatant was added to the pellet and resuspended.

Isolation of mitochondria. Mitochondria were isolated according to the standard protocol of differential centrifugation. The brain was diced by scissoring in the isolation medium: 350 mM mannitol, 50 mM sucrose, 20 mM Hepes-Tris, 1 mM EDTA, 0.1% BSA and homogenized for 4-5 passes. The homogenate was then centrifuged at 4000*g* for 5 min. The supernatant was collected and stored on ice. The pellet was suspended with 8 ml of the isolation medium without BSA, homogenized for the second time, and supplied with 1.25 ml of digitonin-containing medium (3 mg/ml). After 5-min incubation on ice, the material was centrifuged at 2500*g* for 5 min. The pellet was removed, and the supernatant was combined with the first supernatant and centrifuged at 10,000*g* for 10 min. The pellet was resuspended in the medium without digitonin and BSA and repeatedly centrifuged at 12,000*g* for 10 min. The washed pellet was resuspended in 200 μl of the medium. Protein concentra-

tion was determined by the Biuret method using BSA as a standard.

Determination of respiration activity and mitochondrial membrane potential ΔΨ. Respiration and mitochondrial membrane potential were registered simultaneously with a platinum Clark electrode and using a TPP-selective electrode. The registration medium contained 120 mM KCl, 10 mM Hepes-Tris, 0.2 mM EDTA, 5 mM malate, 5 mM glutamate, 2 mM K_2HPO_4, 0.5 μM TPP, pH 7.4.

Detection of cytochrome *c* in homogenates and supernatants. The cytochrome *c* content in homogenates and supernatants was determined by registration of differential optical spectra of reduced versus oxidized cytochrome forms [7]. The spectra were registered on an Aminco DW2000 (USA) spectrophotometer at the temperature of liquid nitrogen. Several dithionite crystals were added to the "sample" cell, and ferricyanide (5 mM) was added to the "reference" cell.

Chemicals. RNase A from Sigma (USA), cyclosporin A from Fluka (Switzerland), and rotenone and FCCP from Serva (Germany) were used in the study. Other chemicals were of highest purity grade commercially available.

Statistics. The experiments were done in 3-4 replicates. Results of typical experiments are presented in the figures. Significance of difference was determined using Student's *t*-test with various dispersions (Excel software).

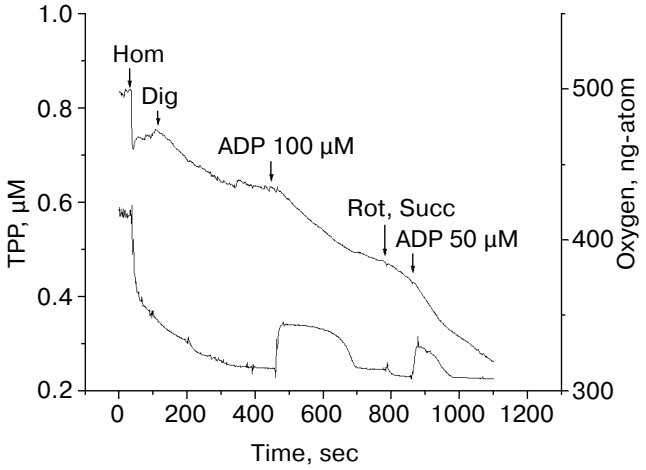

Fig. 1. Mitochondrial respiration (upper curve) and ΔΨ (lower curve) in homogenates of brain cortex tissue after incubation under anoxia for 1 h. Additives: homogenate (Hom); ADP; digitonin, 150 μg/ml (Dig); rotenone, 1 μM (Rot); succinate, 10 mM (Succ).

RESULTS

Dynamics of changes in functional activity of mitochondria. Figure 1 shows the results of measurements of mitochondrial respiration rate and ΔΨ values in homogenates prepared from brain tissue slices after the incubation under anoxic conditions for 1 h. After the incubation, the slices were washed with the incubation medium cooled to 0°C, rapidly (0.5-1 min) homogenized, and placed into a polarographic cell for simultaneous measurement of respiration rate and potential. The time between opening of the tubes and beginning of measurement of mitochondria was no more than 3 min. In accordance with [8], digitonin was added to the cell to improve penetration of synaptosomes for oxidative phosphorylation substrates. It is seen from Fig. 1 that respiratory and phosphorylation activities are maintained after 1 h of incubation under anoxic conditions. Respiration is stimulated after addition of ADP. The magnitude of respiratory control was 3.9 ± 0.2 on malate and 2.3 ± 0.3 on succinate as oxidation substrates; efficacy of oxidative phosphorylation was close to maximum: ADP/O value was 2.9 ± 0.1 and 1.8 ± 0.1 in respiration on the substrates of the first and second complexes, respectively (means ± standard deviations for three independent experiments are given). One can see that the incubation of the slices under anoxic conditions for 1 h does not result in significant damage to the mitochondrial functional activity. When the duration of the incubation increased, the activity of the oxidative phosphorylation system suffered first of all. After incubation of slices for 4 h under anoxic conditions, the respiration of homogenates became insensitive to ADP addition and the mitochondria lost their ability to generate membrane potential (data not shown). Addition of cyclosporin A at high concentrations (100 μM) into the incubation medium did not prevent the membrane potential dissipation.

A similar pattern was observed in mitochondria isolated from slices after 24 h of their incubation under anoxia (Fig. 2a). The respiratory activity of these mitochondria was substantially maintained on oxidation of substrates of both the first and second respiratory complexes (Table 1). During the second and third days of the incubation the oxidation of substrates of the first respiratory complex ceased (Figs. 2b and 2c), the succinate oxidase activity was retained (Table 1). Respiratory characteristics of control mitochondria corresponded to norm: ADP/O value was 2.9 ± 0.1 and 1.9 ± 0.1 on malate and succinate oxidation, respectively; the respiratory control magnitude on malate oxidation was 3.8 ± 0.4; and on succinate oxidation was 2.7 ± 0.3 (means ± standard deviations for three independent experiments are given) (Fig. 2d). Our study of the glycine effect on mitochondrial functions demonstrated that the presence of this substance in the incubation medium under anoxia for 48 h does not prevent ΔΨ dissipation of the mitochondrial membrane and disturbance of malate oxidase activity in the mitochondria (data not shown). However, some minor but significant activation of rotenone-sensitive respiration was detected when glycine was directly added into the mitochondrial suspension (into the cell for the

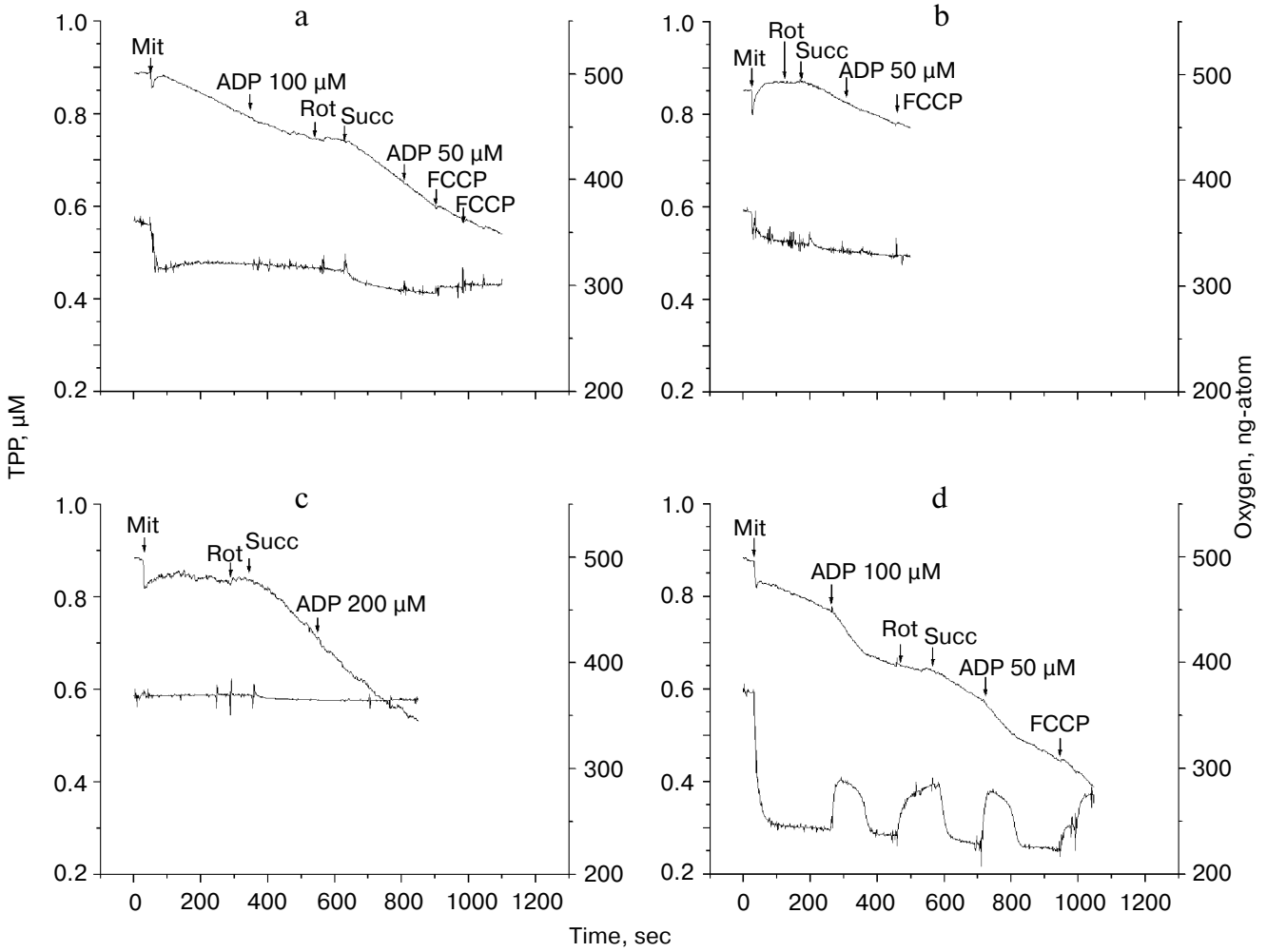

Fig. 2. Respiration (upper curves) and ΔΨ (lower curves) in mitochondria isolated from brain cortex slices after incubation under anoxia for 24 (a), 48 (b), and 72 h (c), and control mitochondria (d). Additives: mitochondria (Mit); ADP; rotenone, 1 μM (Rot); succinate, 10 mM (Succ); FCCP, 0.1 μM (FCCP).

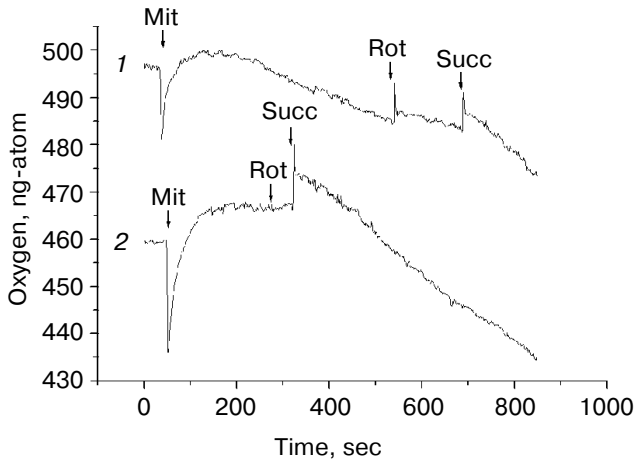

Fig. 3. Effect of glycine on the respiration of mitochondria isolated from brain tissue incubated for 48 h under anoxia: *1*) 5 mM glycine was added into the medium before the beginning of the measurement; *2*) no additions before the beginning of the measurement.

respiration measurement) (Fig. 3 and Table 1). These mitochondria were isolated from slices after incubation for 2 days under anoxia. There was no malate oxidation before the addition of glycine.

Dynamics of outer mitochondrial membrane damage during the incubation of brain cortex tissue under anoxic conditions. We found on homogenates of brain cortex slices that cytochrome *c* is tightly bound with the inner mitochondrial membrane and does not leak out of mitochondria during the first 3 days of incubation in ion-free medium under anoxic conditions. The tissue homogenate was obtained in sucrose medium, centrifuged at 20,000*g* for 10 min, the pellet was resuspended in the same medium, and the cytochrome *c/b* ratio was determined in the suspension. This ratio was unchanged relative to the control (Table 2, a-c, medium M1). Cytochrome *c* was not detected in the supernatant under these conditions. It must be emphasized that the homogenization is done in medium with low ionic strength, and – under these conditions – the cytochrome *c* binds to the inner mitochon-

Table 1. Maximum respiratory rates of uncoupled mitochondria (ng-atom O/min per mg protein) isolated from brain cortex tissue after various incubation duration under anoxia

Incubation duration, h	Respiratory rates on the following substrates			*N*
	glutamate and malate	glutamate and malate + glycine	succinate	
0 (control)	70 ± 11	no measurements	64 ± 9	3
24	39 ± 21	no measurements	46 ± 24	3
48	0 ± 2	15 ± 7*	43 ± 28	3
72	0 ± 2	no measurements	51 ± 25	3

Note: Means ± standard deviations are presented; *N* is a number of independent experiments.
* Respiratory rates (on glutamate and malate as substrates) of mitochondria isolated from the tissue after 48-h incubation in the presence and absence of glycine differ significantly ($p < 0.05$).

Table 2. Dynamics of outer mitochondrial membrane damage during the development of apoptosis under anoxia by the release of cytochrome *c* from intermembrane space under high ion strength of the medium for homogenization of the brain slices

Homogenate type	Ratio of absorption maximum peaks for cytochrome *c* (550 nm) and cytochrome *b* (560 nm)	
	low ionic strength medium (M1)	high ionic strength medium (M2)
a) Homogenate obtained from the control brain tissue	1.47 ± 0.01	1.47 ± 0.01
b) Homogenate obtained from the brain tissue incubated for 24 h in the low ionic strength medium	1.47 ± 0.01	1.16 ± 0.03*
c) Homogenate obtained from the brain tissue incubated for 72 h in the low ionic strength medium	1.47 ± 0.01	0.94 ± 0.02*
d) Homogenate obtained from the brain tissue incubated for 72 h in the low ionic strength medium in the presence of 5 mM glycine	no data	0.85 ± 0.03*

Note: Mean ± standard deviation for three independent experiments is presented. Controls are homogenates obtained from intact brain tissue under low and high ionic strength, as well as homogenates obtained from brain cortex after incubation under anoxia in the low ionic strength medium.
* Presented data differ significantly from the control ($p < 0.01$).

drial membrane due to the electrostatic interactions with negatively charged phospholipids [9, 10]. Another pattern is observed when the homogenization is done in a medium with high ionic strength. Under such conditions, the ionic bond between cytochrome *c* and the inner membrane surface is drastically weakened, and the cytochrome *c* leaks away from the intermembrane space after the breakage of the outer membrane. The breakage of the outer membrane occurs after one day of the incubation, resulting in the decrease in the cytochrome *c*/*b* ratio in the pellet (Table 2, b, medium M2). Appearance of cytochrome *c* in supernatant is then detected (data not shown). The observed effect is markedly enhanced on the third day of the incubation (Table 2, c, medium M2). Cyclosporin A added at high concentration (100 μM) into the incubation medium of the slices does not prevent this effect (data not shown). These experiments suggest that cytochrome *c* does not leak away from mitochondria when the slices are incubated in ion-free medium under anoxia, but breakage of the outer membrane occurs. The experiments repeated in the presence of 5 mM glycine in the incubation medium demonstrated that glycine does not prevent the permeabilization of the outer mitochondrial membrane under anoxia (Table 2, d, medium M2).

Dynamics of internucleosomal DNA fragmentation. Internucleosomal DNA fragmentation is one of the hallmarks of apoptosis [11]. The formation of such fragments is clearly detected by electrophoresis. The Fig. 4 shows an electrophoregram of DNA preparations isolated from brain cortex slices after incubation under anoxia for 72, 96, and 120 h. It is seen from the figure that a distinct typical "apoptotic ladder" is observed after 72 h of incuba-

Fig. 4. Electrophoregram of DNA isolated from brain cortex after 72-120 h of incubation under anoxia. M, molecular marker (100-1000 bp); *1-3*) incubation for 72, 96, and 120 h, respectively.

tion, and a significant portion of the DNA remains in high molecular form. After 96-120-h incubation, the extent of DNA internucleosomal decay increases, and the amount of high molecular weight DNA decreases in parallel. A series of experiments separated by a substantial time range demonstrated that the dynamics of DNA fragmentation could vary significantly. However, a substantial amount of high molecular weight DNA remains to the third day in all the cases. Besides, Fig. 4 shows that DNA decay typical for necrosis with the formation of random fragments occurs apart the internucleosomal fragmentation. This fact demonstrates that apoptotic and necrotic processes are activated simultaneously in brain cortex tissue under anoxic conditions.

Glycine added to the incubation medium at the concentration of 5 mM significantly retards the destruction of nuclear DNA (Fig. 5). It is significant that glycine mainly inhibits the internucleosomal DNA fragmentation, whereas the number of random length DNA fragments formed in the system is not changed significantly. Hence, glycine selectively regulates the apoptosis process only.

Alteration of ultrastructure of brain cortex tissue after three days incubation under anoxia. Our study on ultrastructure of intact rat brain isolated and fixed by the submerged technique directly after sacrifice of the animal demonstrates typical structure of nerve tissue well described earlier [12]. It is obvious that this fixation technique gives broadening of intracellular spaces that is typical for the cortex (Fig. 6a). Various elements of nerve tis-

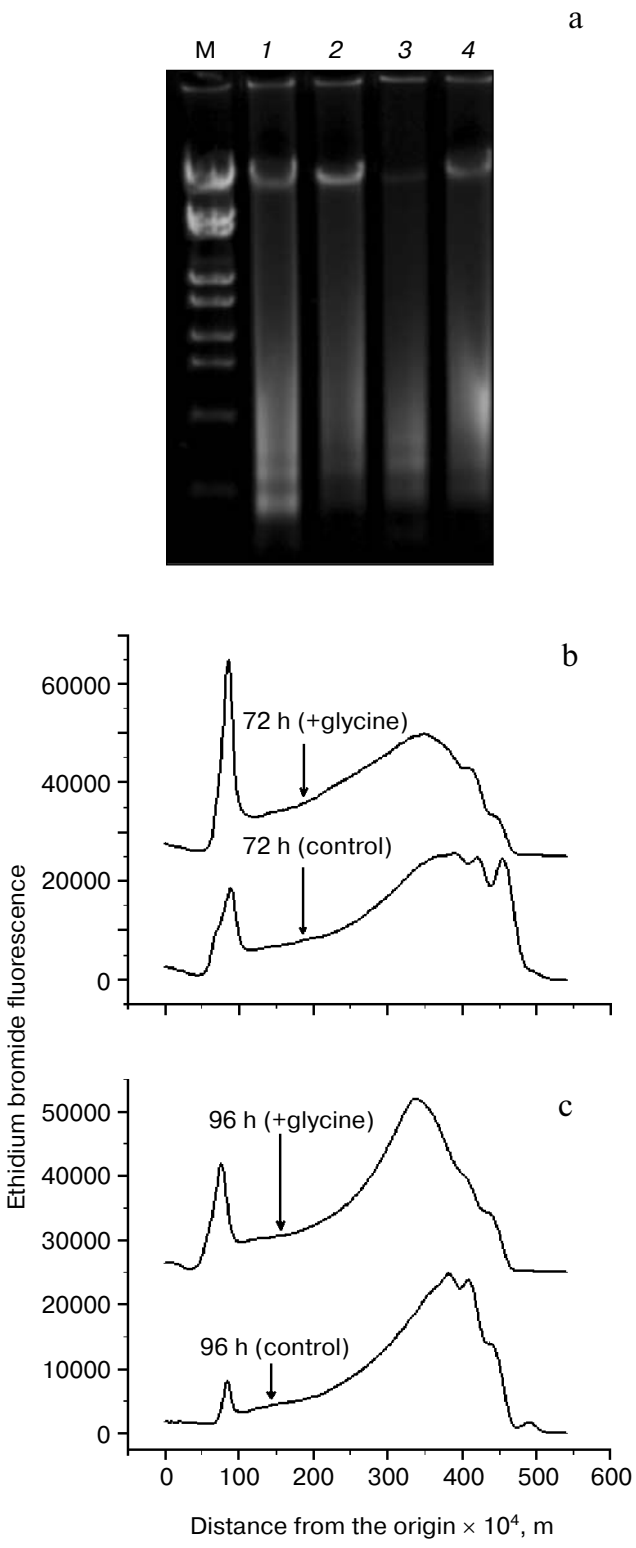

Fig. 5. Inhibition of DNA destruction in brain cortex slices in the presence of glycine. a) DNA electrophoregram: *1*) incubation for 72 h without additives; *2*) incubation for 72 h with 5 mM glycine; *3*) incubation for 96 h without additives; *4*) incubation for 96 h with 5 mM glycine; M, molecular marker (λ DNA/BssT1I (StyI)). b, c) Densitograms of the electrophoregrams (72 and 96 h of incubation, respectively).

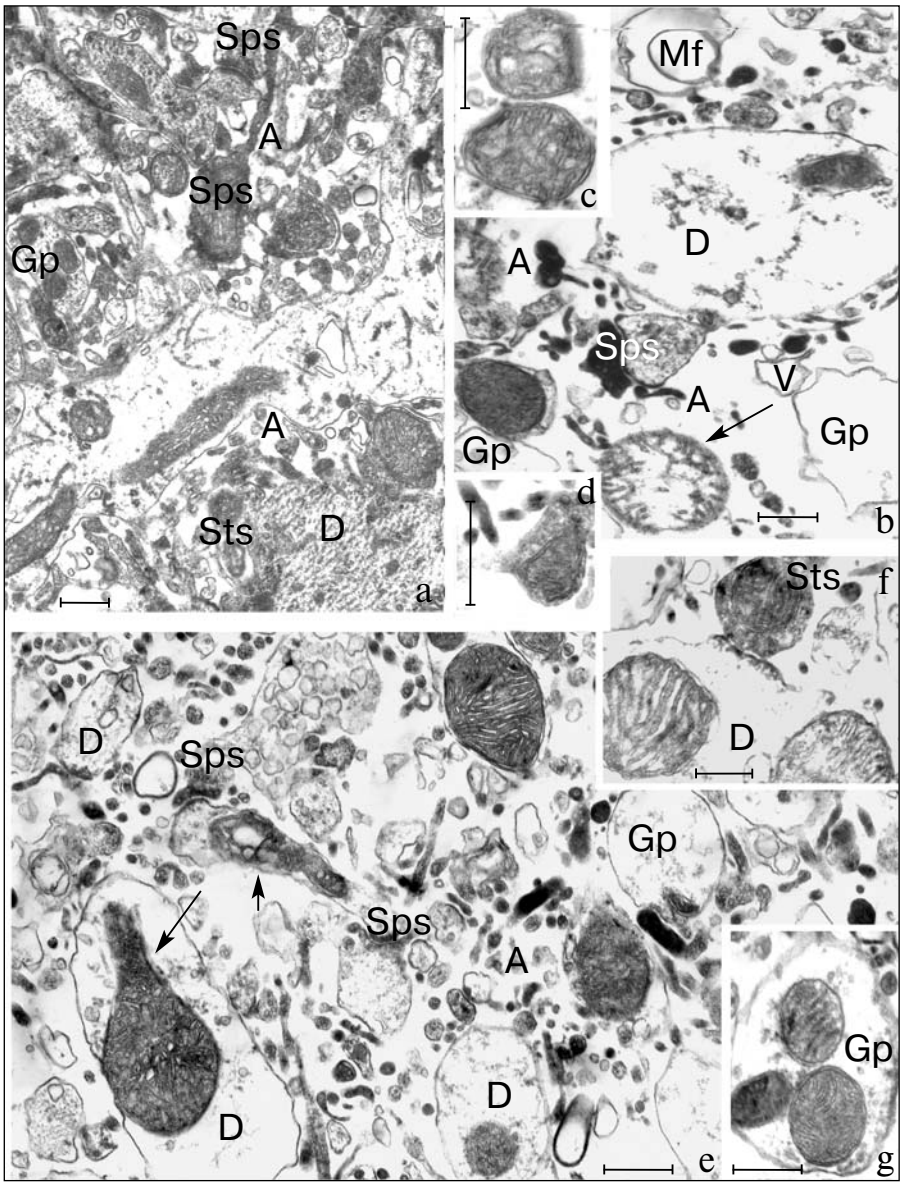

Fig. 6. Ultrastructure of rat brain cortex before and after prolonged incubation under anoxic conditions. a) Intact preparation (submerged fixation of brain slices just after sacrifice of the animal), used for identification of dendrite, axonal (presynaptic), and glial mitochondria. b) Preparation after 72 h incubation under anoxia. General photo, displaying substantial damage to nerve tissue: lysis of plasma membranes in processes, appearance of myelin figures, amplitude of intercellular spaces, the presence of mitochondria inside them (arrow) and a large number of pleiomorphous vacuoles. In the mitochondrial structure, their cristae are subjected to degeneration of various extent, matrix is either clear or condensed, with the appearance of irregular inclusions inside, cristae disappear or vacuolize with distortion of their form. Such pattern is typical not only for dendrite, but also for glial and axonal mitochondria, the ultrastructure of which is shown in insets: in glial process (c) and in one of few retained satisfactory structure in synaptic bud (d). e) Preparation after 72 h incubation under anoxia in the presence of glycine. General photo demonstrating satisfactory state of the nerve tissue and its elements – dendrites, synapses of various types, and glial processes. All the profiles retain their integrity, although intercellular spaces are substantially swollen. Most mitochondria have intact structure. Dumb-bell (arrow) mitochondrial profiles are seen, evidence for anoxic stress, as well as mitochondria with condensed matrix and swollen cristae (vertical arrow). Various state of mitochondria is demonstrated in insets, condensed and orthodox, in the same dendrite and in a synaptic bud on its surface (f), as well as in glial process (g). In this process, a mitochondrion with condensed cristae localized laterally, and a mitochondrion with electron dense matrix with single cristae more typical for intact glial cells are revealed along with a mitochondrion of virtually intact appearance. Denotations: D, dendrite; Sps, spinule synapse; Sts, stem synapse; Gp, glial process; A, non-myelinized areas of axons; Mf, myelin figure; V, vacuole. Scale, 0.5 μm.

sue are easily identified: dendrites, numerous synapses of spinule and stem types, glial processes, and mitochondria inside them. The latter are different in size. The mitochondria are very large in dendrites, are of prolonged form and oriented along the axis of dendrite, and their matrix is moderately electron-dense and cristae are of spontaneous orientation, with more frequent directivity from edges to the center into the depth of the matrix. The

intracristal space is transparent. Glial mitochondria are of medium sizes, they are 1.5-2.0 times smaller than those of dendrites. Cristae are arranged transversely, and the matrix is moderately dense. Synaptic mitochondria are small. They are three and more times smaller than in dendrites, cristae are inconsiderable in number, they are swollen, and the matrix is very electron dense. Small, moderately dense round or prolonged extended profiles are rather numerous. They represent cross-sections of non-myelinated axon sites; some are terminated on dendrite stem or on spinule with synaptic buds. Cytoskeleton of the processes persists well. It is represented by numerous microtubules oriented along the long axis of dendrites. The density of their packing varies—possibly this is associated with the nature of the neuron in which they are localized. The cytoskeleton is represented by interstitial filaments in glial processes.

Ultrastructure of brain cortex site after incubation for 3 days under anoxia at 20°C is represented in Fig. 6b. It is easily observable that the tissue is substantially damaged, and it is in a pathological state. The following observations are evidence for this fact: almost empty profiles of cross-sections of processes, breaks and lacks of membranes, appearance of myelin figures, numerous empty vacuoles of various irregular form, and thin round or prolonged electron dense profiles identified as non-myelinized sites of axons and axon terminals. Generally, mitochondria and synaptosomes inside them are not seen because of extremely dense cytoplasm. These are dark type degenerated axons of neurons. Some mitochondria are located directly in intercellular space outside the cytoplasm of processes, which is evidence for breaks of cytoplasmic membranes usually appearing in the course of necrosis. The lack of microtubules in dendroplasm and other cytoskeleton elements in glial processes is notable. The mitochondrial ultrastructure in dendrites is drastically altered. Cristae are partially lost in some of them, releasing empty broad hollows. In others, cristae are virtually merged with dark matrix and can be scarcely identified. There is no intracristal space in this case. Similar signs of degeneration are seen in mitochondria of glial processes and synaptic buds in the rare cases when they can be identified (Fig. 6, c and d). The formation of electron dense mitochondria is observed, as well as the accumulation of electron dense substance around the periphery of outer mitochondrial membrane. These alterations in mitochondria are usually observed in the course of apoptosis [13, 14].

The ultrastructural state of nerve tissue after prolonged incubation under anoxic conditions in the presence of glycine strikingly differs from the preceding pattern. The surveillance photo (Fig. 6e) shows that general preservation of the tissue and distinct elements such as dendrites, synapses, and glial processes is satisfactory. In spite of broadened intercellular spaces, all profiles of various natural processes are of intact configuration, plump, osmotically active appearance. Plasma membranes are continuous, and breaks are rare. Elements of cytoskeleton are often seen in the openings of processes, microtubules are seen in dendroplasm, and filaments in glial processes. It is worth noting that a portion of dendrites degenerates, as evidenced by vacuolization of dendroplasm. Vacuoles representing possibly degenerated dendrite profiles can be seen in intercellular spaces. Axons possessing electron density and structure close to normal are numerous, along with dark type degenerated axons. They are often terminated with synaptic buds well identified due to the presence of synaptosomes preserving their normal morphology. Mitochondrial behavior is representative under the incubation with glycine. A substantial portion of mitochondria in dendrites is of normal appearance. Along with these mitochondria, others are seen with partially vacuolated and swollen cristae, containing simultaneously electron dense structures formed of crista membranes associated pair wise (or more). Also mitochondria are seen to have round inclusions ringed by single membranes (not shown). Mitochondria of different ultrastructural organization can be seen inside the same dendrite profile (Fig. 6f). As this takes place, mitochondria in synaptic buds localized on the surface of dendrite or dendrite spinule and usually possessing dense matrix have intact appearance. Several mitochondrial profiles can be present in glial processes, each of them has distinct structure: with typical electron dense matrix, strictly cross-oriented, and randomly localized cristae. Mitochondrial matrix is moderately electron dense, and cristae are virtually unswollen.

DISCUSSION

In this study processes developing under prolonged anoxia in brain cortex are characterized, dynamics of DNA decay is determined, character of impairment of mitochondrial energy system is defined, and morphological changes in tissues occurring under the experimental conditions are described. In the second step of the work, glycine at high concentrations is shown to slow apoptotic processes. Induction of two parallel processes—apoptosis and necrosis—occurs in brain cortex under the conditions of our experiment. After 3 days of incubation, internucleosomal DNA fragmentation, a sign of apoptosis, is observed, and also DNA decay to random length fragments that is typical for necrosis is noted in parallel. DNA decay occurs relatively slowly.

Results of our electron microscopic studies are consistent with the results obtained in the study of DNA decay. Degeneration to dark type axons of neurons was revealed after 3 days of incubation of the tissue under anoxia. Mitochondria were present in intercellular space, which occurs after breakage of plasma membranes, and this is typical for necrosis. At the same time, we detected in glial processes the electron dense mitochondria [13,

15] and mitochondria lacking cristae in which electron dense substance is concentrated along the perimeter of the outer membrane. These changes in mitochondria are typical for apoptosis [13, 14]. The study of dynamics of mitochondrial energy processes in the brain cortex under anoxia demonstrated that the mitochondrial system of oxidative phosphorylation is completely retained for 1 h as a minimum, which is several times longer than the time range of preservation of the brain functional activity under anoxia. It is significant that the succinate oxidase fragment of the electron transport chain is very stable and retains its activity for more than 3 days under anoxic conditions. These properties of mitochondria may play a substantial role in tissue regeneration after temporary impairment of circulation in some brain areas.

The synthesis of ATP and the generation of transmembrane electric potential on the inner mitochondrial membrane are less stable; they are totally impaired (dissipated) after several hours of incubation under anoxia of the brain cortex tissue. The mitochondria of brain cortex also lose their ability to oxidize malate, the Krebs cycle substrate, after 48 h of incubation.

Study of the effect of glycine on the resistance of the oxidative phosphorylation system demonstrated that this substance added into the tissue incubation medium has no influence on the dynamics of mitochondrial energy impairment. However, a respiratory stimulation was observed (Fig. 3), when 5 mM glycine was added to the experimental sample cell with mitochondria isolated from heart tissue after 48 h incubation under anoxia. This respiration can be totally inhibited with rotenone, an inhibitor of the first complex of the respiratory chain. As we mentioned above, malate oxidase activity in these mitochondria is absent. Obviously, the activity of the first complex of the respiratory chain is partially preserved, and NADH, the substrate of the first complex, is formed from glycine by the reaction of oxidative decarboxylation [16]. Apparently, it is the formation of NADH upon glycine oxidation that mediates the observed respiratory stimulation (Fig. 4).

The influence of glycine on internucleosomal DNA fragmentation is presented in Fig. 6. One can see from this figure that glycine substantially slows down internucleosomal DNA fragmentation. This effect is specific, since the rate of DNA decay into random fragments (that corresponds to necrosis) remains almost constant.

The data on DNA decay are consistent with the results of electron microscopic studies, which demonstrated that in the presence of glycine a large portion of mitochondria in glial processes possesses moderately electron dense matrix and virtually unswollen cristae. In this case mitochondria devoid of cristae, which are typical for apoptosis, are virtually absent. Obviously, anoxia induces a special type of apoptosis in cortex tissue, which does not involve cytochrome *c*. Cytochrome *c* does not leak from the mitochondrial intermembrane space into the cytoplasm, in spite of damage to the external mitochondrial membrane, which is a barrier for cytochrome *c* translocation to the cytoplasm (Fig. 5b). Unlike apoptosis, occurring for instance in the heart under anoxia/reoxygenation [17], or Bnip3-dependent apoptosis occurring in cardiomyocytes under anoxia [18], the brain apoptosis is not inhibited by cyclosporin A, the inhibitor of the nonspecific pore. Its development apparently does not involve opening of the nonspecific pore.

It seems that in the given case we are faced with apoptosis typical for nerve tissue, the occurrence of which is associated with hyperactivation of glutamate receptor [1, 2]. This supposition is confirmed by the experimentally observed sensitivity of this apoptosis to high concentration of glycine, which dissipates hyperactivation of glutamate receptors arising under anoxic conditions [3, 19].

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