EFFECT OF IN VITRO ANOXIA ON CALCIUM METABOLISM IN THE CEREBRAL CORTEX

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UDC 612.825.015.31:546.41/-06:612.232

KEY WORDS: anoxia; mitochondria; microsomes; synaptosomes; membrane-bound Ca^{++}.

Insufficiency of the oxygen supply leads to definite changes in metabolism and functions of the brain neurons, the mechanisms of which have not been adequately studied. During the investigation of this problem attention must undoubtedly be concentrated on the study of changes in activity of the regulators of intracellular processes, which include not only other secondary messengers, but also Ca^{++} ions. In the modern view, Ca^{++} is an important, but at the same time, a "dangerous" messenger, for an excessive increase of its concentration in the cytosol may lead to profound damage to the cells [11]. It has been suggested that disturbances of intracellular Ca^{++} metabolism and of activity of Ca^{++}-dependent processes in various types of anoxia play a key role in structural damage and functional impairment of brain neurons [13, 14]. Changes in the concentration of membrane-bound Ca^{++} in structures of the cerebral cortex, developing rapidly in anoxia, have recently been discovered in vivo [1].

To determine the particular features of the disturbance of Ca^{++} homeostasis on cessation of the oxygen supply in different compartments of neurons, it was decided to study subcellular fractions of cortical neurons in vitro.

EXPERIMENTAL METHOD

Subcellular fractions (synaptosomes, mitochondria, and microsomes) were isolated from rabbit cerebral hemispheres. Fractions of synaptosomes and mitochondria were isolated by the usual method [4, 5]. Microsomes were obtained from the postmitochondrial supernatant by ultracentrifugation for 1 h at 105,000g. Mitochondria were incubated in the usual medium [3], but depending on the experimental conditions the NaCl concentration in the medium varied from 10 to 30 mM. Synaptosomes were placed in medium of the following composition (in mM): KH_2PO_4 1.24, Tris-HCl 20 (pH 7.4), glucose 10, $CaCl_2$ 1.3, NaCl 124, KCl 5 (in some experiments the NaCl and KCl concentrations were changed to 99 and 30 mM, respectively). The incubation medium for microsomes contained 10 mM KCl, 10 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, and 0.5 mM ATP. To assess changes in the concentration of Ca^{++} bound mainly with hydrophobic components of the membranes of the fractions studied (Ca^{++}_{mb}), a fluorescent chelate probe was used, namely chlortetracycline (CTC), in a concentration of 10 μm [7, 8]. Measurements of the intensity of fluorescence of Ca^{++}–CTC–membrane complexes in the 520 nm region were made on an Aminco-Bowman spectrofluorometer with excitation wavelength of 410 nm. Anoxic conditions for the subcellular test fractions (for comparison with aerobic incubation) were created by saturating the medium beforehand with nitrogen.

Uptake of Ca^{++} by synaptosomes incubated in medium with radioactive calcium was determined in a series of experiments. After incubation, uptake of ^{45}Ca by the synaptosomes was stopped by addition of cold medium containing Ca^{++} ions and 5 mM EGTA, after which the samples were filtered without delay through Whatman GF/B filters, which were then washed in 10 ml of incubation medium. Radioactivity of the samples was determined by scintillation spectrometry on a Beckman LS 9000 instrument.

Department of Neurochemistry, Medical Research Center, Polish Academy of Sciences, Warsaw. Group for Regulation of Brain Neuron Functions, I. P. Pavlov Institute of Physiology, Academy of Sciences of the USSR, Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR S. N. Golikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 3, pp. 261-264, March 1988. Original article submitted April 22, 1987.

0007-4888/88/0003-0301$12.50

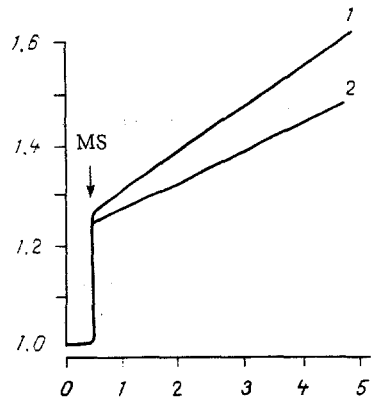

Fig. 1

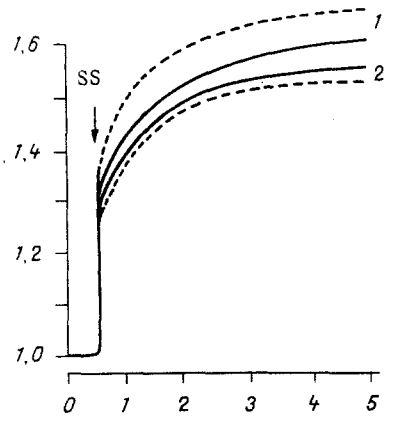

Fig. 2

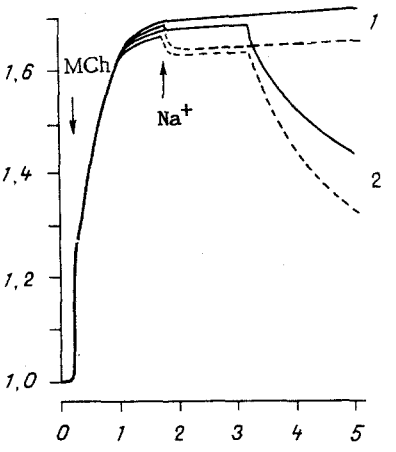

Fig. 3

Fig. 1. Dynamics of Ca^{++}_{mb} concentration in microsomes (MS) during incubation in anoxic medium (2) compared with the control (1). Here and in Figs. 2 and 3: abscissa, time (in min); ordinate, intensity of fluorescence of Ca^{++}–CTC-membrane complexes (in relative units).

Fig. 2. Dynamics of Ca^{++}_{mb} concentration in synaptosomes (SS) during incubation in control (1) and anoxic (2) media differing in their KCl concentration. Continuous line — 5 mM KCl; broken line — 30 mM KCl in medium.

Fig. 3. Dynamics of Ca^{++}_{mb} concentration in mitochondria (MCh) during incubation in control (1) and anoxic (2) media differing in their NaCl concentration. Continuous line — 10 mM KCl in medium; broken line — NaCl concentration increased to 30 mM (arrow indicates addition of Na^+).

EXPERIMENTAL RESULTS

After introduction of the subcellular fractions into anoxic incubation medium disturbances of the Ca^{++}_{mb} concentration were found in each of them. However, the rate and degree of the changes in the Ca^{++}_{mb} concentration in hydrophobic regions of the membranes of these fractions differed in character. In the synaptosomes and microsomes a rapid decrease (between 30 and 60 sec of anoxia) was found in fluorescence of CTC–Ca^{++}_{mb} (Figs. 1 and 2) compared with the control. Meanwhile, in the mitochondria this process was discovered much later (Fig. 3).

Dependence of the disturbance of the Ca^{++}_{mb} concentration in the synpatosomes and mitochondria on changes in concentration of monovalent cations (K^+ and Na^+, respectively) in the incubation on medium, which are known to take place during anoxia in the cerebral cortex in vivo [2], was studied in various experiments. During the first few minutes of anoxia the extracellular K^+ concentration in the cortex in vivo was close to its initial value (3-5 mM), but later it rose sharply, to coincide with the development of strong depolarization of the neurons, and by the 5th-8th minute of anoxia it had reached 30-60 mM. Meanwhile, during anoxic depolarization, the intracellular Ca^{++} concentration increased by several times. Accordingly, in different versions of the experiments, incubation media were used with 5, 30, and 60 mM K^+ (for synaptosomes) and 10 and 30 mM Na^+ (for mitochondria). With an increase in the K^+ concentration in the anoxic medium from 5 to 30-60 mM, the process of Ca^{++}_{mb} released in the synaptosomes was found to be significantly intensified. For instance, in medium with a low K^+ concentration the intensity of CTC–Ca^{++}_{mb} fluorescence fell by 4-6%, whereas in medium with high K^+ it fell by 22-30% compared with the control (Fig. 2).

In the series of experiments with a low Na^+ concentration in the medium (10 mM), a decrease in CTC–Ca^{++}_{mb} fluorescence of the mitochondria was found only 6-7 min after the beginning of exposure to anoxia (Fig. 3). Later, with lengthening of this exposure, a progressively greater decrease of CTC–Ca^{++}_{mb} fluorescence was recorded. In another series, at the 3rd minute of the experiments, the Na^+ concentration was increased to 30 mM in both normoxic (control) and anoxic incubation medium. In both cases there was some decrease in CTC–Ca^{++}_{mb}

TABLE 1. Effect of Anoxia on ^{45}Ca Uptake by Rabbit Brain Synaptosomes (M ± m, n = 4)

Incubation conditions	^{45}Ca uptake, nmoles/mg protein/min	
	5 mM KCl	30 mM KCl
Normoxia	7,01±0,14	8,54±0,31
Anoxia	5,84±0,42*	7,49±0,25*

Legend. *$p < 0.05$.

fluorescence in response to anoxia, connected with the known factor of Na^+–Ca^{++} exchange in the mitochondria. Meanwhile the very marked (more so than in the previous series of experiments) reduction of CTC–Ca^{++}_{mb} fluorescence also began to appear 6-7 min after addition of the mitochondria to the anoxic medium (Fig. 3).

In the experiments with ^{45}Ca-loading of the synaptosomes a small decrease in uptake (compared with the control) of extracellular Ca^{++} by these fractions was observed in anoxic incubation medium containing both low and high K^+ concentrations (Table 1).

On the basis of the results and of data in the literature the mechanism of anoxic disturbance of intracellular Ca^{++} metabolism in the cerebral cortex can be represented as follows. In response to deficiency of the oxygen supply a decrease in the concentration of the Ca^{++} fraction bound with hydrophobic regions of the intracellular membranes takes place rapidly in cortical structures in vivo (during the first few tens of seconds) [1]. However, the Ca^{++} containing regions of membranes of different compartments of cortical neurons differ in the level of their sensitivity to anoxia. Reduction of CTC–Ca^{++}_{mb}-fluorescence begins much earlier in synaptosomes and in structures of the endoplasmic reticulum than in mitochondria.

The rapid anoxic changes in the Ca^{++}_{mb} concentration in the neurons in vivo, and also those in synaptosomes and microsomes in vitro, correlate with early changes in the intracellular redox state with accumulation of reducing equivalents and they precede disturbances of homeostasis of other ions, of neuronal activity, and of ultrastructure [2]. Reduction of CTC–Ca^{++}_{mb}-fluorescence may be evidence not only of a decrease in the concentration of membrane-bound Ca^{++}, and an increase in their concentration in the cytosol, but also of the passage of Ca^{++} from hydrophobic into hydrophilic regions of the membranes [8]. This process is evidently one of those significantly involved in the development of disturbances of ionic permeability, plasmalemma excitability, and activity of many intraneuronal enzymes due to deficiency of the oxygen supply.

During long-term exposure to anoxia a marked decrease takes place in the extracellular concentration of Ca^{++} with their accumulation in the neurons [6, 9]. This process may lie at the basis of the mechanism of the pathological action of the "toxic Ca^{++}-overloading" which, according to some investigators, can lead to death of brain neurons [12-14]. At the same time, however, the absence of any increase in the inflow of ^{45}Ca into the synaptosomes in anoxic medium in vitro, in agreement with data in the literature [10], merits attention. The much better state of preservation of the ultrastructure of nerve endings than of the body and dendrites of the neuron during long-term total cortical ischemia [2] may perhaps be connected with the characteristics of the changes in Ca^{++} metabolism described above.

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CORRELATION BETWEEN DESTRUCTIVE AND REPARATIVE PROCESSES IN RAT CEREBRAL CORTICAL NEURONS AFTER BURN TRAUMA

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UDC 617-001.17-092.9-07:
616.831-091.81-003.9

KEY WORDS: neurons; rat cerebral cortex; RNA metabolism; electron-microscopic autoradiography.

When the time course of a pathological process is described the reparative phase is generally regarded as one which develops, not at the beginning of the process, but at a certain late, or even the final stage [2]. However, from the theoretical point of view it is evidently correct to consider that repair is triggered by injury and, consequently, it must take place immediately after the beginning of the action of the destructive factor or after a certain latent period necessary for the particular reparative response to be activated. Investigation of these concepts on a model of hepatitis [3] showed that the reparative response, expressed as intensification of RNA synthesis in the hepatocytes located at the periphery of the lobule, develops simultaneously with the spreading of necrosis in hepatocytes at the center of the lobule. Another reparative response leading to much more prolonged and stable compensation and to stimulation of DNA synthesis develops in the preserved cells later, after the lapse of sufficient time for completion of all processes preparatory for DNA synthesis.

Considering the unique character of the biological and pathological processes in the brain, we decided to study the development of reparative and destructive processes in neurons. It was recalled that, unlike in the liver, which can regenerate through division of its cells, neurons have an exclusively intracellular type of regeneration. To investigate the chosen problem the method of electron-microscopic autoradiography was used, for it enables the intensity of RNA synthesis, an important reparative process, to be compared with ultrastructural changes characterizing damage to and repair of the cell.

EXPERIMENTAL METHOD

Noninbred albino rats weighing 180 g were anesthetized with ether and a stage IIIB-IV burn covering 20% of the body surface was inflicted. RNA synthesis was investigated in intact animals (control) and 4, 15, 75, and 144 h after burning (five animals at each time). Altogether 25 animals were used. For electron-autoradiographic investigation of RNA synthesis, the animals were anesthetized with ether and the RNA precursor 5-^{3}H-uridine (specific activity 26 Ci/mmole) was injected into the brain substance (the cerebral cortex in sensomotor area PA^m) through a special needle. The labeled precursor (50 μCi) was dissolved in 0.05 ml of Ringer's solution. Pieces of cortex were removed 3 h after injection of the label and fixed in a 2.5% solution of glutaraldehyde in phosphate buffer, pH 7.4. For the next 24 h the fragments were washed with buffer, with repeated change of the solution, and postfixed in 1% OsO_4 solution. After dehydration in alcohols, the fragments were embedded in an Epon mixture. Light-microscopic autoradiographs were first prepared on semithin sections and the region of cerebral cortex for ultramicrotomy of layer V was chosen on the

Department of Pathological Anatomy, M. F. Vladimirskii Moscow Regional Clinical Research Institute. Department of Pathological Anatomy, A. V. Vishnevskii Institute of Surgery, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR D. S. Sarkisov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 3, pp. 264-267, March 1988. Original article submitted June 15, 1987.

 0007-4888/88/0003-0304$12.50